Claim 62:	A host cell transformed or transfected with the isolated nucleic acid molecule of claim 54.
Claim 63:	A host cell transformed or transfected with the isolated nucleic acid molecule of claim 55.
Claim 64:	A host cell transformed or transfected with the isolated nucleic acid molecule of claim 56.
Claim 65:	The host cell of claim 62, wherein said cell is a fibroblast.
Claim 66:	The host cell of claim 63, wherein said cell is a fibroblast.
Claim 67:	The host cell of claim 64, wherein said cell is a fibroblast.
Claim 48:	The host cell of claim 62, wherein said cell is a mammalian cell.
Claim 49:	The host cell of claim 63, wherein said cell is a mammalian cell.
Claim 50:	The host cell of claim 64, wherein said cell is a mammalian cell.

REMARKS

Claims 54-67 will be pending. These claims replace the previously submitted claims. They recite complete hybridization conditions.

Please note that with the exception of reference sequence SEQ ID NO: 18, claims 54-67 parallel claims found allowable in Serial Nos. 09/583,850 and 09/597,179. To this end, any office action which follows that rejects claims without including a complete discussion of why the examiner – who is in the same art unit as are the examiners who allowed the prior case – is differing from art unit practice, will be considered incomplete, the matter will be taken up with the supervisory primary examiner, and a petition will be filed.

Claims 39 & 47 were NOT drawn to the same subject matter as claims 37 and 45. Careful review of claims 54, 56, 62 and 64 will show this. To facilitate review, claim 37 and now claim 54 recites a tumor rejection antigen precursor. Claim 39 and now claim 56 recites a tumor rejection antigen, NOT the precursor. This is not a slight difference in wording, as the specification clearly explains the differences between these molecules.

With respect to the rejection of old claim 41, now claim 58, the examiner is again referred to the parallel prosecutions. Also see pages , lines , of the specification.

The examiner has rejected all of the claims as allegedly lacking utility. The examiner states that,

> "No disclosure, however, is found in the specification whether SEQ ID NO: 18 is overexpressed in melanoma cell line as compared to normal cells. No disclosure is found in the specification whether the SEQ ID NO: 18 is expressed in tissue, and even if SEQ ID NO: 18 is expressed in tissue whether it is overexpressed in cancer tissue as opposed to normal tissue."

The above quotation is simply the "tip of the iceberg" of a long, involved, and confusing rejection.

SEQ ID NO: 18 is also known as MAGE-6, as the specification makes clear. MAGE-6 is expressed in tumor cell lines and in tumors. Appended hereto are several references. Note DePlaen, et al, Immunogenetics 40:360-369 (1994). Please see page 367. With the exception of testis, there was no expression in normal tissue. Tumors, including LB175, LB271 (lung cancer & melanoma), showed expression. Also see Rosenberg, et al, Biological Therapy of Cancer, page 499, a copy of which is attached.

The molecule is expressed as a protein, which is then processed to peptides (tumor rejection antigens), that form complexes with MHC molecules, and provoke cytolytic T cells. See Zorn, et al, Eur. J. Immunol 29:602-607 (1999). Tanzarella, Canc. Res. 59:2668-2674 (1999); Tatesumi, et al, J. Exp. Med. 196(5):619-628 (2002), as well as U.S. Patent Nos. 5,928,938, 6,265,215 and 6,323,028.

One does not secure CTLs without peptides. And one does not secure these peptides unless the protein form which they are derived is present. And one does not secure these proteins unless the gene is expressed. A review of the attachments show that MAGE-6 is expressed in tumor cells and it is translated into a protein. Hence, the examiner's argument, to the extent it is understood, is rebutted by the facts, which include (i) evidence of expression in tumors, (ii) evidence of overexpression in tumors as compared to normal tissues, (iii) expression of protein, and (iv) processing of proteins to peptides. The utility rejection over pages 6-10 is clearly improper and should be withdrawn.

Turning now to the written description rejection, it is first of all pointed out that Regents University of California v. Eli Lilly is NOT relevant here. In the case cited by the examiner, no DNA was presented which satisfied the claim. In contrast, SEQ ID NO: 18 is so presented. Further, with respect to the "complement" language, the application as a whole states that tumor rejection antigen precursors are molecules large enough to be processed to smaller ones. The

claims are drawn to molecules which encode such larger molecules. They cannot be considered to consist of a "few" nucleotides.

Finally, the examiner's position is diametric to the guidelines which examiners are supposed to follow. In the event the examiner is not familiar with the Interim Examination Guidelines for Written Description, applicants attach a copy of Example 9, which is "on all fours" with the claims.

If the examiner maintains this rejection, and does not fully explain how (i) the guidelines are not satisfied, and (ii) how the Eli Lilly case is relevant in view of the clear disclosures in the specification, then this matter will be taken up with the supervisory primary examiner.

With respect to the "enablement" rejection at page 13, the examiner admits that this is linked to the rejection under 35 U.S.C. §101. This has been rebutted. Hence, the lack of enablement rejection is moot.

To the same end, the scope rejection does not consider what is claimed. Only nucleic acid molecules which encode tumor rejection antigen precursors, their fragments, or tumor rejection antigens, are claimed. All are defined in the specification such that they would be cognizable to the skilled artisan. The rejection is misplaced. Again, please refer to the allowed claims in the companion cases. As for the rejection over pages 15 et seq, the examiner is again directed to the evidence of protein expression provided herewith.

As for the prior art rejection, it is clearly improper. The art relied upon by the examiner consists of 10 nucleotides. At most, this could encode 3 amino acids. As is explained in the specification, tumor rejection antigens are at least 8 amino acids long. They are encoded by nucleotides at least 24 nucleotides long. Fragments of tumor rejection antigen precursor are larger than this. Hence, a 10 nucleotide stretch does not meet the claim. Further, since the rejected claim calls for a nucleic acid molecule which encodes a polypeptide, applicants would like to know where the start codon is in the cited art.

Allowance of this application is proper, and is urged.

Respectfully submitted,

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Registration No. 30,946

VIA FIRST CLASS MAIL

I hereby certify that this correspondence is being deposited with the U.S. Postal Service as FIRST CLASS MAIL in an envelope addressed to the Commissioner of Patents and Trademarks, Washington, D.C. 20231 on November 22, 2002.

Laurie Olds

Laurie Olds

LUD-5353.7 DIV JEL/NDH (10016357)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Applicant	:	Beatrice GAUGLER et al.
Serial No.	:	09/583,848
Filed	:	May 31, 2000
For	:	ISOLATED NUCLEIC ACID MOLECULES CODING FOR TUMOR REJECTION ANTIGEN PRECURSOR MAGE-6 ND USES THEREOF
Art Unit	:	1642
Examiner	:	M. Davis

November 22, 2002

Hon. Commissioner of Patents
and Trademarks
Washington, D.C. 20231

SHOWING OF CHANGES

Page 27, last two lines:

The sequence for the E antigen precursor gene has been determined and is presented herein and at SEQ ID NO: 7.

Page 36, lines 20-26:

To do this, and using standard protocols, cells normally insensitive to anit-E/CTLs were incubated with the synthetic peptides derived from Exon 3.1. Using the CTL lytic assays described supra on P815A, and a peptide concentration of 3mM the peptide of SEQ ID NO: 26, i.e., Glu-Ala-Asp-Pro-Thr-Gly-His-Ser-Tyr was shown to be best. The assay showed lysis of 30%, indicating conferrins of sensitivity to the anti-E CTL.

Respectfully submitted,

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PRINCIPLES AND PRACTICE OF THE BIOLOGIC THERAPY OF CANCER

Third Edition

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TABLE 2. EXPRESSI N IN TUMOR SAMPLES OF MAGE-TYPE GENES ENC DING T-CELL ANTIGENS

Histologic Type	Percentages of Tumors Expressing: MAGE-A1	MAGE-A2	MAGE-A3	MAGE-A4	MAGE-A6	MAGE-A10	MAGE-A12	BAGE	GAGE-1,2,7	GAGE-3-6,8	LAGE-1	NY-ESO-1
Melanoma	25	52	55	18	59	21	34	12	29	41	33	17
Primary lesions	46	70	74	28	72	47	62	31	41	50	41	35
Metastases												
Lung carcinoma	44	42	48	59	53	50	28	9	39	42	27	17
Squamous cell carcinoma	49	47	44	35	49	40	33	13	36	45	44	40
Adenocarcinoma	31	38	51	53	58	35	27	6	26	28	35	24
Head and neck squamous cell carcinoma												
Bladder carcinoma	14	11	16	23	19	33	10	3	3	3	47	47
Superficial (<T2)	32	43	57	45	57	33	34	26	25	35		
Infiltrating (≥T2)											20	10
Esophageal carcinoma	53	53	63	74	68	40	26	6	44	44		
Squamous cell carcinoma							40	0	20	20	20	20
Adenocarcinoma	20	40	20	20	20	10	5	0	15		27	33
Prostate carcinoma	18	18	18	0	23	7	8	0	17		24	35
Sarcoma	8	15	8	15	15	0	16	12	10		23	23
Breast carcinoma	19	9	13	6	15	0	11	0	0		0	0
Colorectal carcinoma	0	13	17	11	22	0	0	0	0		0	0
Renal cell carcinoma	5	0	0	2	0	0	0	0	0	4	0	0
Leukemia and lymphoma	0	0	0	1	0							
Myeloma						0	0	0	0	0	0	0
Stages I–II	0	0	0	0	0	7	15	15	44	44	52	30
Stage III	30	19	31	12	33							

Note: Expression was measured by reverse transcriptase-polymerase chain reaction on total RNA using primers specific for each gene.
From Brasseur F, Brussels Branch of the Ludwig Institute for Cancer Research, with permission.

renal carcinoma and leukemia. This picture is consistent for all the MAGE-type genes, even those from different families. Also consistently observed is a higher frequency of expression in metastatic versus primary melanoma, and in infiltrating versus superficial bladder carcinoma. This coordinated expression suggests that a common mechanism controls the activation of MAGE-type genes. Transfection studies of the MAGE-A1 promoter have shown that it can exert transcriptional activity in cells that do not express the gene, suggesting that the transcription factors capable of activating *MAGE-A1* are ubiquitous (38). Transcriptional control of *MAGE* genes therefore does not involve specific transcription factors. Indeed, it was found later to rely on the methylation of the promoter. The MAGE-A1 promoter contains binding sites for transcription factors of the Ets family, which are essential for its activity (38). These sites contain CpG dinucleotides, which are methylated in cells that do not express the gene, thereby preventing the binding of the Ets factor (39). Treatment of tumor cells or fibroblasts with the demethylating agent 5'-aza-2'-deoxycytidine activates gene *MAGE-A1* (40). This is also true for most MAGE-type genes. A global demethylation of the genome is often observed in tumor cells and is correlated with tumor progression. It is also correlated with *MAGE-A1* expression, which therefore appears to be a random consequence of this genome-wide demethylation (39). This provides an explanation for the higher frequency of MAGE expression in advanced cancers. It also explains the expression of MAGE-type genes in male germ cells, which also have reduced levels of methylated DNA (41).

Among the *MAGE* genes not listed in Table 2, *MAGE-B2* is expressed in 45% of non–small-cell lung cancers and in 22% of melanomas, whereas *MAGE-C1* is expressed in 46% of melanomas and 18% of bladder cancers (17,27).

OTHER MECHANISMS PRODUCING SHARED TUMOR-SPECIFIC ANTIGENS

Two antigens that have been characterized on human melanoma also represent specific shared antigens, but their presence on tumor cells results from different genetic mechanisms. The first one is due to the activation of a cryptic promoter located in an intron of the gene coding for *N*-acetylglucosaminyltransferase V, an enzyme involved in protein glycosylation (42). An abnormal transcript is produced, containing the 5'-end of this intron and the following exon. This transcript is totally absent from normal cells but is present in approximately 50% of melanomas. The peptide recognized by the CTL is encoded by the intronic part of this transcript and is therefore strictly tumor specific. The reason why this cryptic promoter is active in melanoma and not in normal cells is unknown. The second antigen results from an incomplete splicing of the transcript of *TRP2*, a gene encoding a

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A MAGE-6-encoded peptid is recognized by expand d lymphocytes infiltrating a spontaneously regressing human primary melanoma lesion

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In recent years, experiments based on the *in vitro* stimulation of either autologous peripheral blood lymphocytes or tumor-infiltrating lymphocytes with melanoma cells have shown that distinct members of the large MAGE gene family encode tumor-associated antigenic peptides. However, little is still known about natural anti-MAGE responses *in vivo*. We have studied a case of spontaneously regressing human melanoma, hypothesizing that in this unique situation, the host immune system had developed an efficient cytotoxic T lymphocyte (CTL) response against the cancer cells. Amongst the dense tumor infiltrate, certain clonal populations of T cells were shown to be amplified, thereby suggesting that an antigen-driven selection had occurred at the tumor site. One of the expanded tumor-infiltrating lymphocytes was shown to be a Vβ13$^+$ CD8$^+$ CTL displaying a strong and selective cytotoxic activity against the autologous melanoma cells. Here we show that this cytotoxic T cell clone recognizes a MAGE-6-encoded peptide. MAGE-6 is therefore the fourth gene of the MAGE family shown to encode antigenic peptide recognized by T cells. Together, these data provide further evidence that T cell responses against MAGE antigens may naturally develop *in vivo*.

Key words: Tumor-infiltrating lymphocyte / Regressive melanoma / Immunosurveillance / Tumor antigen / MAGE

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1 Introduction

A primary regressive human melanoma was used as a potential model to study naturally elicited anti-tumor immune responses [1]. Initial investigation of the regressive lesion revealed the presence of expanded clonal populations within the infiltrating T lymphocytes, strongly suggesting an antigen-driven selection at the tumor site. Two of these populations were further studied [2, 3]. The most predominant one, bearing a TCR Vβ 16 segment, is described in the accompanying article [4]. The other is characterized by the expression of the unique Vβ13.1-WGGD-Jβ1.1 rearrangement. Although less amplified at the tumor site than the former Vβ16$^+$ tumor-infiltrating lymphocyte (TIL) clone, it was found to represent 30 % (6 out of 20 sequences) of the Vβ13$^+$ T lymphocytes while the corresponding Vβ13 subfamily appeared to be represented almost 2 times more in the tumor than in the PBL [1, 2]. Following *in vitro* cloning, functional studies indicated that this Vβ13$^+$ clone, designated 5G, was a CD8$^+$ CTL displaying a strong and selective cytotoxic activity against the autologous tumor cells [2]. Here we demonstrate than this TIL clone 5G recognizes a MAGE-6-encoded peptide presented by HLA-A3402.

[I 18534]

Abbreviations: TIL: Tumor-infiltrating lymphocyte **ORF:** Op n reading frame **LCL:** Lymphoblastoid cell line

0014-2980/99/0202-602$17.50+.50/0

2 Results

2.1 Clone 5G is restricted by HLA-A3402 molecules

The patient under study had been serologically typed as HLA-A3, -A10, -B14, -B35, -Cw4 and -Cw8. In earlier studies, blocking experiments performed with anti-HLA typing polysera suggested that HLA-B14 molecules restricted the recognition of M10 cells by the *in situ* amplified Vβ13$^+$ TIL clone 5G [2]. To identify the recognized antigen, multiple rounds of cotransfection experiments were then attempted using a cDNA library prepared with the M10 cell line and the HLA-B14 cDNA. Results were negative, suggesting that either the relevant cDNA was not included in th library or that HLA-B14 was not restricting the interaction of clone 5G with the tumor cells. We therefore cloned th five additional HLA class I transcripts and used an alternate strategy to

characterize the restriction element. The six HLA transcripts cloned from M10 cells were transfected, using lipofectamine, into 17 allogeneic melanoma cell lines which were then tested for their capacity to stimulate TNF secretion by clone 5G. As shown in Table 1, the transfection of HLA-A3402 generated a signal in 11 out of 17 melanoma cell lines. These results strongly suggested that the 5G TIL clone is restricted by HLA-A3402 as no signal was found with the additional HLA alleles including HLA-B14. In addition, they indicated a frequent expression of the recognized antigen.

2.2 MAGE-6 cDNA encodes the antigen recognized by clone 5G

Expression screening into COS-7 cells was then performed using HLA-A3402 cDNA and the PB1g library divided into pools of about 100 bacterial clones. Ten pools out of the 465 tested were found to be positive in duplicate cultures. Pool 456 that conferred the highest reactivity was subscreened to monoclonality. As shown in Fig. 1, cDNA clone 92.3, thus obtained, induced TNF release by 5G lymphocytes when transfected into COS-7 cells with HLA-A3402. The sequence of this 1.5 kb long cDNA clone revealed an open reading frame (ORF) of 972 bp, which is 100 % identical to that of MAGE-6 mRNA (GENBANK; accession: D32076).

2.3 Identification of the MAGE-6 peptide recognized by clone 5G

To establish which region of the cDNA encodes the antigenic peptide, fragments of the MAGE-6 transcript were cloned and functionally tested. Two clones were generated, starting at positions Ala 154 and Asp 241 and corresponding to the cDNA clone 92.3 deleted of fragments included between the 5' end and either a Hind III or a BamH I restriction site, respectively. A third clone, start-

Table 1. Stimulation of clone TIL 5G by melanoma cell lines transfected with HLA transcripts

Tumor cell lines	TNF release by TIL5G[a]					
	Transfected HLA[b]					
	A0301	A3402	B1401	B3508	Cw0401	Cw0802
Me 13923	–	+++	–	–	–	–
Me 1402	–	+++	–	–	–	–
M102	–	–	–	–	–	–
M95	–	+	–	–	–	–
Me 8530	–	–	–	–	–	–
M96	–	+	–	–	–	–
M82	–	+++	–	–	–	–
HTB71	–	+	–	–	–	–
M25	–	–	–	–	–	–
DR.	–	–	–	–	–	–
M12	–	+	–	–	–	–
M60	–	–	–	–	–	–
1.54	–	++	–	–	–	–
M17	–	+++	–	–	–	–
M16	–	+	–	–	–	–
VE.	–	–	–	–	–	–
BL.	–	+++	–	–	–	–

a) TNF release was measured by the WEHI 164/13 bioassay. Results are reported in equivalent TNF-β: <1.5 pg/ml (-); 1.5–12.5 pg/ml (+); 12.5–50 pg/ml (++); >50 pg/ml (+++).

b) Tumor cell lines were transfected with pCDNA3 vector containing the different HLA cDNA, using lipofectamine as explained in Sect. 4.5. Forty-eight hours later, 2500 CTL were added to the culture. Supernatants were assessed for their TNF content after 6 h of coculture.

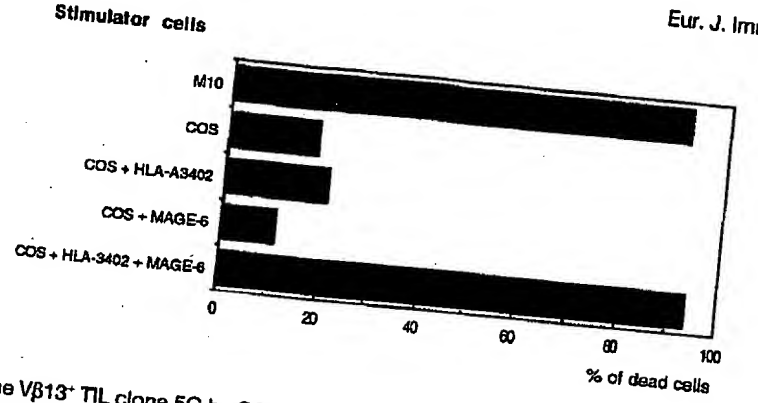

Figure 1. Stimulation of the Vβ13⁺ TIL clone 5G by COS-7 cells transfected with 10 ng/well of MAGE-6 cDNA and 100 ng/well of HLA-A3402 cDNA inserted into a pCDNA3-expressing vector. Control stimulator cells include the autologous melanoma cells M10, untransfected COS-7 cells and COS-7 cells transfected either with HLA-A3402 cDNA alone or MAGE-6 cDNA alone. After 6 h of coculture, supernatants were assessed for their effect on TNF-sensitive cells (WEHI 164/13).

ing at position Leu 278, was obtained by cloning an Apa I-Apa I restriction fragment. As illustrated in Fig. 2, these three clones transferred reactivity to TIL 5G when transfected into COS cells together with HLA-A3402, suggesting that the antigenic peptide is encoded in the Apa I cDNA fragment. As its expression in COS-7 cells is controlled by an internal AUG codon, it could be concluded that the antigenic peptide is localized within the 25 amino acid long sequence downstream of Met 290. The 15 possible overlapping 11 amino acid long peptides included in this latter sequence were then tested in a chromium release CTL assay using the autologous B-EBV cells (lymphoblastoid cell line, LCL) pulsed with the different peptides as targets. Following a 10 μM pulse, peptide, MVKISGGPRIS was the only one out of the 15 tested to induce LCL lysis by clone 5G (data not shown). We then investigated whether the nonapeptide MVKISGGPR and decapeptide MVKISGGPRI could also be recognized by clone 5G. As shown in Fig. 3, nona-, deca- and hendecapeptide were antigenic. However, the nonapeptide was 100 times more efficient in sensitizing LCL to lysis by clone 5G. Half-maximum lysis was obtained at concentrations of 50 nM, 10 μM and 5 μM for nona-, deca- and hendecapeptides, respectively.

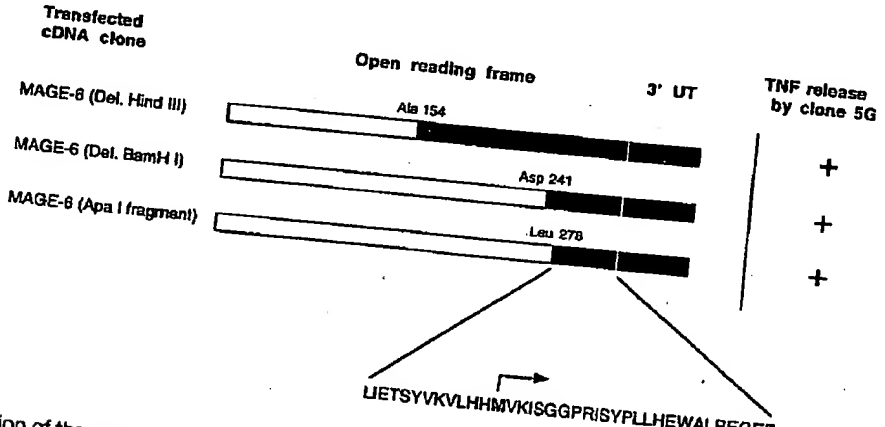

Figure 2. Identification of the region of MAGE-6 cDNA which encodes the antigenic peptide. Fragments of MAGE-6 cDNA, indicated by black boxes, were cloned in pCDNA3 expressing vector and transfected into COS-7 cells together with HLA-A3402 cDNA. Starting residues of the cDNA fragments are indicated. After 48 h of expression, transient transfectants were tested for their ability to stimulate TIL clone 5G in a TNF release assay. For fragm nts marked +, supernatants induced a lysis of >90 % of the WEHI cells. The amino acid sequence of the ORF region which transfers the reactivity to the Vβ13⁺ TIL clone corresponding to the Apa I fragment (codons 278–314) is indicated together with the first methionine at codon 290 (broken arrow).

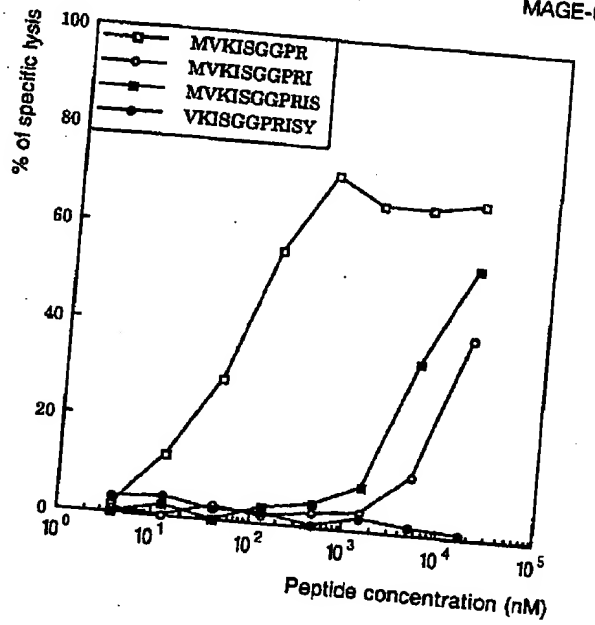

Figure 3. Lysis by the Vβ13⁺ TIL clone 5G of autologous LCL cells incubated with MAGE-6-encoded peptides. LCL cells were ^{51}Cr-labeled, pulsed for 1 h with synthetic peptides at the indicated concentrations, then incubated with Vβ13⁺ cells at an E/T ratio of 10/1. Chromium release was measured after a 4-h period. An irrelevant MAGE-6 hendecapeptide (codons 291-301) was used as a control (closed circles).

3 Discussion

In the present study, we show that the CD8⁺ cytolytic TIL clone 5G, expanded at the site of the primary spontaneously regressive melanoma lesion, recognizes a MAGE-6-encoded peptide presented by HLA-A3402 molecules on the autologous tumor cells.

In 1991, MAGE-1 [5] was defined as the first gene encoding melanoma antigen in human and led to the further characterization of a large family of related genes with at least 11 additional members named MAGE-2 to MAGE-12 and located in the q terminal region of chromosome X [5, 6]. Amongst them, only MAGE-1, -2, -3, -4, -6 and -12 were shown to be transcribed [6]. They are expressed in multiple tumor cell lines of different histological types, in the testis and for some of them in the placenta (MAGE-3 and -4) but not in other normal adult tissues (6–8). To date, their functional role remains unknown. Regarding MAGE-6 specifically, its nucleotide sequence is 98 % identical to that of MAGE-3 and its expression pattern appears similar to that of the ther members of the MAGE family. In previous studies, MAGE-1, -3 and -12 genes have been shown to enc de melanoma-associated antigenic peptides [9–13]. It is of note, however, that the reported MAGE-12 peptide is encoded by a sequence identical to its MAGE-3 counterpart [12]. The peptide MVKISGGPR described here is unique to the MAGE-6 sequence. Accordingly, MAGE-2, MAGE-3 and MAGE-12 transcripts cloned from M10 cells failed to be recognized by TIL 5G when contransfected into COS-7 cells with HLA-A3402 (data not shown). With the demonstration of a fourth member of the MAGE family involved in natural immune responses, the present study further supports the importance of MAGE-encoded antigens in anti-tumor immunity.

The MAGE antigenic peptides were originally characterized using PBL stimulated *in vitro* in the presence of autologous tumor cells. This provided evidence for the existence of MAGE-specific CTL precursors within circulating T cells in melanoma patients. Subsequent studies suggested that such specific precursors might also infiltrate tumors such as melanoma [14] or breast cancer [15]. By demonstrating the *in situ* amplification of MAGE-specific T cells at the primary melanoma site [2], our series of experiments provides additional support for the involvement of such effector lymphocytes in anti-tumor responses.

As described previously [16], the patient developed two subsequent metastases 3 and 4 years, respectively, after the primary tumor was surgically removed. Characterization of the T cell infiltrate revealed that the TIL clone 5G was still present in these two secondary lesions [16]. Moreover, the two cell lines derived *in vitro* from both metastases were effectively killed by this Vβ13⁺ effector, indicating the persistence of the MAGE-6 antigen. Notably, these results indicate that the presence of the MAGE-6-specific T lymphocytes had been sustained over a 4-year period. However, analysis of the Vβ13 transcripts at the metastatic sites revealed that the 5G cells were poorly represented *in situ* with substantially less redundancy in the corresponding Vβ sequences as compared with the primary lesion [16].

Together, these data show that a MAGE-specific T cell response was developed and maintained in the course of a human melanoma. This response was not dominant in the context of the initial tumor regression and was found to still be present after 3 and 4 years, when rapidly progressing metastatic lesions eventually emerged. These findings are in contrast with those reported in the accompanying article, where the dominant response was found t be directed against a mutated antigen and no longer detected in the two subsequent metastases [4]. As a whol , ur series of experiments provides additional knowledge about the position of MAGE gene prod-

ucts as melanoma-associated antigens. Further studies will be required to gain a better insight into the actual contribution of such natural responses to the control of the tumor expansion *in vivo*.

4 Materials and methods

4.1 General remarks

Patient characteristics and clinical course, DNA sequencing, TNF bioassay, peptides and CTL assay are described in the accompanying article [4].

4.2 Cell lines and TIL clone

Allogeneic melanoma cell lines were obtained from Dr. F. Triebel, INSERM U. 333, Institut Gustave Roussy, Villejuif, France (DR., M12, 1.54, VE. and BL.), from Dr. F. Jotereau, INSERM U. 211, Institut de Biologie, Nantes, France (M102, M95, M96, M82, M25, M60 and M17), from Dr. G. Parmiani, Division of Experimental Oncology, Istituto Nazionale Tumori, Milano, Italy (Me1402, and Me8530) and from the American Type Culture Collection (HTB-71). The additional cell lines are described in the accompanying article together with their culture procedures [4]. Clone 5G has been described previously [2].

4.3 Construction of the cDNA library

The PB1g cDNA library used in these experiments was prepared as described in the accompanying article [4] with minor modifications. The first strand of cDNA was synthesized with the superscript choice system for cDNA synthesis (Gibco-BRL, Gaithersburg, MD), according to the manufacturer's instructions and using a clamped oligo-dT primer containing a NotI restriction site: AAC CCG GCT CGA GCG GCC GCT TTT TTT TTT TTT TTT TT(GAC) (ACGT). Newly synthesized double-stranded cDNA was size fractionated and only the largest fraction (>1400 bp) was ligated into pCDNA3 vector (Invitrogen). Plasmid DNA was extracted by the alkaline lysis method from 465 pools of 100 cDNA.

4.4 Cloning of the HLA alleles from the M10 cell line

Five of the six classical HLA class I alleles were cloned from the autologous tumor cell line cDNA library by Southern blot. Plasmid DNA (200 ng) from each pool of the PB1g fraction was transferred onto nylon membranes (Bioprobe, Montreuil, France) and fixed covalently by UV exposure using a stratalinker (Stratagene). Membranes were then hybridized with a 533-bp ^{32}P-labeled, probe corresponding to the HLA-A0301 α1-domain (ApaI-AvaI restriction fragment of the genomic HLA-A0301 clone p44/2; [17]) using standard procedures at a hybridization temperature of 65 °C. Each positive pool was subscreened to monoclonality and the corresponding insert was sequenced. Transcripts including the entire ORF and corresponding to HLA-A0301, -A3402, -Cw0401 and -Cw0802 were thus obtained. The HLA-B3508 transcript was not isolated directly from the library using this method but later obtained by reverse transcription PCR and subsequent cloning. Complete sequencing of the six inserts showed that there was no mutation according to their corresponding allelic sequences recorded in GENBANK (data not shown).

4.5 Transfection of tumor cell lines and CTL stimulation assay

Transfection of melanoma cell lines was performed using lipofectamine (Gibco-BRL) by a previously described method [18] with minor modifications. Tumor cells (10^4 cells/well in a flat-bottom 96-well microplate) were transfected by a mixture composed of 100 µl RPMI 1640 without serum, 200 ng of HLA plasmid DNA, 1 µl of lipofectamine and incubated overnight. RPMI 1640/20 % FCS (100 µl) were then added to the mixture and incubated an additional 24 h. The following day, the medium was discarded and TIL were added at a concentration of 2500 cells/well in 100 µl RPMI 1640/10 % human AB serum. After a further incubation period of 6 h, 50 µl of supernatant was used for TNF bioassay on WEHI 164/13 cells.

4.6 Expression screening of the cDNA library in COS-7 cells

Expression screening was performed as described in the accompanying article [4]; however, COS-7 cells were transfected with 100 ng of pCDNA3 containing HLA-A3402 cDNA and 100 ng of a pool of the PB1g cDNA library.

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Identification of a Promiscuous T-Cell Epitope Encoded by Multiple Members of the *MAGE* Family[1]

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ABSTRACT

One of the major limitations of tumor-specific vaccination is the generation of antigen-loss variants that are able to escape the immune response elicited by a monoantigenic peptide epitope. Here, we report the identification of a new HLA-B*3701-restricted epitope shared by four different members of the *MAGE* family. Peripheral blood lymphocytes isolated from a melanoma patient were stimulated *in vitro* with the autologous HLA-negative melanoma line transfected with autologous HLA B*3701 molecule. This protocol led to the induction of tumor-specific, B*3701-restricted CTLs specific for a peptide epitope encoded by codons 127–136 of the gene *MAGE-1*. The same epitope is also encoded by the homologous region of three other members of the *MAGE* family, *MAGE-2*, *-3*, and *-6*. Consistent with the notion that the peptide encoded by *MAGE-1* codons 127–136 is, indeed, processed from the proteins encoded by all four *MAGE* family members, the CTLs were able to specifically recognize Cos-7 cells cotransfected with HLA-B*3701 and any of these *MAGE* genes. Moreover, the CTLs also recognized a *MAGE-6*-positive melanoma line transfected with the B*3701 molecule. These findings allow the inclusion of a new set of tumor patients into clinical cancer vaccination trials. Furthermore, they suggest that some promiscuous peptide epitopes shared by different members of the *MAGE* family might be less prone to escape the immune response by generation of *MAGE* antigen loss variants.

INTRODUCTION

A number of studies have demonstrated that CTLs recognizing human tumor cells can be isolated from PBLs[3] and tumor-infiltrating lymphocytes of cancer patients (1). An *in vivo* role of such tumor-specific effectors is suggested by the results of several immunotherapy trials, mainly performed in melanoma patients (2–5).

Over the last few years, the use of such tumor-specific effectors has allowed the isolation of several genes encoding tumor antigens (6). According to the pattern of expression in neoplastic and normal tissues, these antigens can be classified into four classes, which have different degrees of tumor specificity and clinical relevance. The first class comprises antigens encoded by genes expressed in various tumors of different histotypes but not in normal tissues, other than testis and placenta, such as *MAGE*, *GAGE*, and *BAGE* (7–9). The second class represents differentiation antigens that are only expressed in melanoma and melanocytes, such as *tyrosinase*, *Melan-A/MART-1*, *gp100*, *TRP-1*, and *TRP-2* (10–15). The antigens belonging to the third class are generated by point mutations in genes that are ubiquitously expressed (16–18). The fourth class of antigens, which has been defined only recently, is represented by *TRP-2-INT2*, an antigen shared between melanomas but not expressed in normal cells of the melanocytic lineage (19).

Tumor antigens belonging to the *MAGE* family have especially elicited considerable interest because six of them, *MAGE-1*, *-2*, *-3*, *-4*, *-6*, and *-12*, are expressed selectively by a significant proportion of primary and metastatic tumors, including melanomas, lung, bladder, ovarian, and breast carcinomas (7, 20–23).

Clinical trials of vaccination, based on the use of antigenic peptides encoded by *MAGE-1* and *-3*, are in progress in patients affected by melanoma and other neoplastic diseases (3). Nevertheless, the use of *MAGE-2*, *-4*, and *-6* proteins as targets for tumor-specific immunotherapy has been hampered by the uncertainty as to whether these proteins can be recognized by specific CTLs of the immune system.

Potential limitations to the wide application of this therapeutic approach are the limited number of characterized CTL epitopes (*i.e.*, tumor antigen peptides and appropriate HLA class I alleles) and the *in vivo* generation of antigen loss variants that are able to escape the immune response elicited by a monoantigenic vaccine (24). Indeed, such clinical protocols apply only to patients carrying a tumor expressing a well-known tumor antigen and a defined HLA allele. Unfortunately, a large majority of cancer patients do not fulfill these including criteria. Therefore, identification of new antigenic determinants is a priority because it would increase the number of patients that could benefit from antitumor vaccination protocols.

Here, we report the identification of a new HLA-B*3701-restricted epitope, encoded by homologous regions of the *MAGE-1*, *-2*, *-3*, and *-6* genes. The use of identical antigenic peptides derived from different highly homologous proteins, in protocols of vaccination, may represent a useful tool to avoid the generation of antigen loss variants. Moreover, this study presents, for the first time, evidence for the existence of human CTL recognizing peptides derived from MAGE-2 and -6 proteins, which now can be included in the list of possible antigens for targeted immunotherapy of neoplastic disorders.

MATERIALS AND METHODS

Cell Lines. The melanoma cell line MSR3-mel was established in our laboratory from a metastatic lesion of patient MSR3 and cultured in IMDM supplemented with 10% FCS. PBLs of this patient were serologically typed as: HLA-A1, A11, B37, B5, Cw6. The melanoma line MZ2-MEL.2.2 ET.1 (HLA-A1, B*3701, Cw6), hereafter referred to as ET1, and the Cos-7 cell line were kindly provided by Prof. T. Boon (Ludwig Institute for Cancer Research, Brussels, Belgium) and maintained in DMEM supplemented with 10% FCS. Me14932 (HLA-A2/A3, B7/Bw50, Cw6/Cw7) was a kind gift of Dr. G. Parmiani (Istituto dei Tumori, Milano, Italy). The B-lymphoblastoid cell line LG2-EBV was kindly provided by Prof. T. Boon, whereas the MSR3-EBV was derived by transformation of peripheral blood B lymphocytes from patient MSR3 with the B95-8 strain of EBV.

Synthetic Peptides. Synthetic peptides were purchased from Primm (Milano, Italy). Peptides were: $MAGE_{127-136}$ (REPVTKAEML), encoded by codons 127–136 of *MAGE-1*, *-2*, *-3*, and *-6* genes; and $M4_{127-136}$ (KELVTKAEML) and $M12_{127-136}$ (REPFTKAEML), corresponding to amino acids 127–136 encoded by genes *MAGE-4* and *-12*, respectively. Peptides were dissolved to 10 mM in DMSO and diluted further in 0.9% NaCl.

Subcloning of the *HLA-B*3701* Allele. Total RNA was prepared from MSR3 PBLs by the RNeasy Total RNA Kit (Qiagen, Hilden, Germany). cDNA corresponding to 300 ng of total RNA was amplified by PCR using a primer pair suitable for specific amplification and directional cloning of the full-length

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[3] The abbreviations used are: PBL, peripheral blood lymphocyte; IMDM, Iscove's modified Dulbecco's medium; mAb, monoclonal antibody; IL, interleukin; LNGFR, low-affinity nerve growth factor receptor; β2-m, β2-microglobulin.

coding region of *HLA-B* alleles (generous gift of Dr. Soo Young Yang, Memorial Sloan-Kettering Cancer Center, New York, NY). The 1.1-kb PCR product was subcloned into the eukaryotic expression vector pcDNA3.1 (Invitrogen Corp., Oxon, United Kingdom). Plasmid clones encoding HLA-B*3701 and -B*52011 (the *HLA-B37* and *-B5* alleles of patient MSR3) were identified using diagnostic restriction enzymes. The *HLA-B*3701* gene was then sequenced to verify the correspondence to the published DNA sequence. This plasmid was called pcDNA3.1/B*3701.

Transfection of Melanoma Cell Lines. Melanoma cell lines were transfected by the calcium phosphate precipitation technique with pcDNA3.1/HLA-B*3701 and selected in G418. Expression of the transfected HLA-B*3701 molecule in stable transfectants was verified by flow cytometry with the HLA-A-, HLA-B-, and HLA-C-specific mAb W6/32.

***In Vitro* Induction of CTL Line 337.** CTL line 337 was obtained using a protocol previously described by others (25), with slight modifications. Briefly, PBLs from patient MSR3 were separated by Ficoll gradient and cultivated (1×10^6–2×10^6 cells/well) with the autologous, irradiated MSR3-B37 melanoma cells (0.5×10^5–1×10^5 cells/well) in 2 ml of IMDM supplemented with 10% human serum, glutamine, and antibiotics. After 3 days of culture, 10 units/ml IL-2 (Chiron, Milan, Italy) and 5 ng/ml IL-7 (Genzyme Corp., Cambridge, MA) were added. Lymphocytes were restimulated weekly with 0.5×10^5 irradiated MSR3-B37 cells and tested in a cytotoxicity assay after three stimulations. After the fifth restimulation, 2×10^6 irradiated LG2-EBV cells were added as feeder cells, and IL-2 was increased to 50 units/ml.

Assay for Cytolytic Activity and Peptide Binding Studies. Lytic activity of the cytotoxic T-cell lines was tested in a chromium release assay as described previously (26). Peptides were tested in chromium release assays: ^{51}Cr-labeled target cells were incubated for 1 h at room temperature in 96-well microplates with various concentration of the peptide before addition of effector cells at a fixed E:T ratio. Binding of peptides $M4_{127-136}$ and $M12_{127-136}$ to the HLA-B*3701 molecule was studied in a competition assay, as described previously (27). As standard peptide, we used peptide $MAGE_{127-136}$ (300 nM), recognized by CTL 337. CTLs were used at an E:T ratio of 30:1.

Production of Subfragments of *MAGE-1*. Subfragments of *MAGE-1* gene (495- and 1072-bp fragments) were obtained by digestion of *MAGE-1* cDNA with *Bgl*II and *Eco*RI. After purification on agarose gel, the fragments were cloned into the pcDNA3.1 plasmid. Clones were isolated, plasmid DNA was extracted and transfected into Cos-7 cells along with the *HLA-B*3701* gene.

Transfection of Cos-7 Cells and IFN-γ Release Assay. Transfection of Cos-7 cells was performed by the DEAE-dextran-chloroquine method (12). Briefly, 1.5×10^4 Cos-7 cells were transfected with 100 ng of plasmid pcDNA3.1/B*3701 and 100 ng of expression vectors containing the cDNA of one of the following genes: *MAGE-1*, *-2*, *-3*, *-4*, *-6*, and *-12*. Transfected Cos-7 cells were tested in a IFN-γ assay after 48 h: 5000 responder CTLs, at day 5 after stimulation, were added in 150 μl of IMDM-10% human serum supplemented with 25 units/ml IL-2. After 24 h at 37°C, 100 μl of supernatant were harvested, and the IFN-γ concentration was measured using a IFN-γ release kit (Genzyme Corp.) according to the manufacturer's recommendations.

Retroviral Vector-mediated Gene Transfer of *HLA-B*3701* into Me14932. The retroviral vector B37-CSM, coding for the HLA-B*3701 molecule of patient MSR3, was constructed as described previously (28). Briefly, the full-length cDNAs coding for the HLA-B*3701 molecule was cloned under the control of the viral long terminal repeat, whereas the truncated form of the human LNGFR (ΔLNGFR) was driven by the SV40 promoter. The ecotropic murine fibroblast cell line GP+E86 was transiently transfected with 30 μg of retroviral construct by standard calcium-phosphate method. Infection of the amphotropic murine packaging cell line GP+env Am 12, by supernatant of 48 h cultures of transfected GP+E86 cells, was performed for 4 h in the presence of 8 mg/ml polybrene. Infected packaging cells were immunoselected for ΔLNGFR expression by magnetic beads (Dynabeads M-450; Dynal A.S., Oslo, Norway) coated with the LNGFR-specific mAb 20.4 (American Type Culture Collection, Rockville, MD). Transduction of Me14932 was performed by cultivation with retrovirus-containing supernatant in the presence of polybrene (8 mg/ml). Five or six rounds of infection of at least 4 h were performed. Efficiency of infection was evaluated by immunofluorescence analysis with the LNGFR-specific mAb 20.4 and with a HLA-Bw4-specific mAb.

RT-PCR Assays. *MAGE-1*, *-2*, *-3*, *-4*, *-6*, and *-12* and β2-*m* cDNAs were detected by PCR amplification. Reaction mixture contained 5 μl of cDNA suspension, 4 μl of a 10 mM dNTPs mixture (containing each dNTP at 2.5 mM), 5 μl of 10× DNA polymerase buffer (Finnzymes Oy, Espoo, Finland), 2 units of DynaZyme DNA polymerase (Finnzymes Oy), and sterile distilled water up to a 50-μl total reaction volume. For oligonucleotide primer sequences and PCR amplification programs, see Weynants *et al.* (Ref. 20; MAGE-1, -2, and -3) and De Plaen *et al.* (Ref. 29; MAGE-4, -6, and -12). β-2m cDNA was amplified using the sense primer β 5′ (5′-AAC CAC GTG ACT TTG TCA CAG C-3′) and antisense primer β 5′ (5′-CTG CTC AGA TAC ATC AAA CAT G-3′). PCR amplification was performed for 30 cycles (1 min at 94°C, 30 s at 56°C, and 2 min at 72°C); the expected length of β-2m amplification product was 230 bp. RNA integrity was tested by PCR with β-actin-specific oligonucleotide primers (30). Samples scored positive when a band of the appropriate size was visible on a agarose gel in the presence of ethidium bromide.

RESULTS

MSR3-B37 Induces an Antigen-specific Immune Response. The melanoma line MSR3 was established from a cutaneous metastasis resected from patient MSR3. Expression of HLA class I alleles by the tumor cells was barely detectable (Fig. 1) and appeared to be inadequate to allow antigen presentation to immune effectors. Indeed, the MSR3 melanoma line failed to induce a cytotoxic response from autologous PBLs (data not shown). The lack of class I cell surface expression by MSR3-mel was not caused by impaired β-2m synthesis because a β-2m-specific mRNA was detected by RT-PCR analysis (data not shown).

To determine whether HLA class I antigen expression could be restored, MSR3-mel cells were stably transfected with cDNA encoding the autologous HLA-B*3701 molecule. After G418 selection flow cytometric analysis showed staining of the transfected MSR3-B37 cell line by the W6/32 mAb (Fig. 1).

To evaluate the presence on the surface of MSR3-B37 line of tumor-specific antigens, the melanoma cells were tested for their ability to induce tumor-specific cytotoxic effectors and for their susceptibility to lysis by these CTLs. Patient's PBLs were *in vitro* stimulated by MSR3-B37 as described in "Materials and Methods." After three rounds of stimulation, the polyclonal cytotoxic T cell line 337 (CTL 337) specifically lysed the MSR3-B37 cell line but not the untransfected MSR3-mel (Fig. 2). Autologous MSR3-EBV cells and PHA-activated T blasts were not recognized (data not shown), suggesting that the epitopes recognized by these CTLs are melanoma-/melanocyte-specific. Indeed, in addition to the autologous melanoma cells, CTL 337 also lysed the HLA-B*3701-positive melanoma line ET1 (Fig. 2), suggesting that one or more shared melanoma antigens are recognized.

These data indicate that HLA class I expression can be restored by transfection of MSR3 melanoma cells and that the melanoma line transfected with the HLA-B*3701 molecule is able to induce a tumor-specific cytotoxic T-cell response.

Identification of the Antigenic Epitope Recognized by CTL 337. To identify the antigen recognized by CTL 337, we evaluated the IFN-γ release of CTL 337 in the presence of Cos-7 cells transfected with plasmid pcDNA3.1/B*3701, along with cDNA encoding six members of the *MAGE* family (*i.e.*, *MAGE-1*, *-2*, *-3*, *-4*, *-6*, and *-12*), some of which are expressed by both MSR3-mel and ET1. CTL 337 specifically recognized Cos-7 cells transfected with *MAGE-1*, *-2*, *-3*, and *-6* (Fig. 3), suggesting that the epitope target of CTL 337 was shared among the four different antigens or that distinct components of the oligoclonal T-cell line were recognizing peptides derived from the four *MAGE* gene products. A low level of IFN-γ was detected in

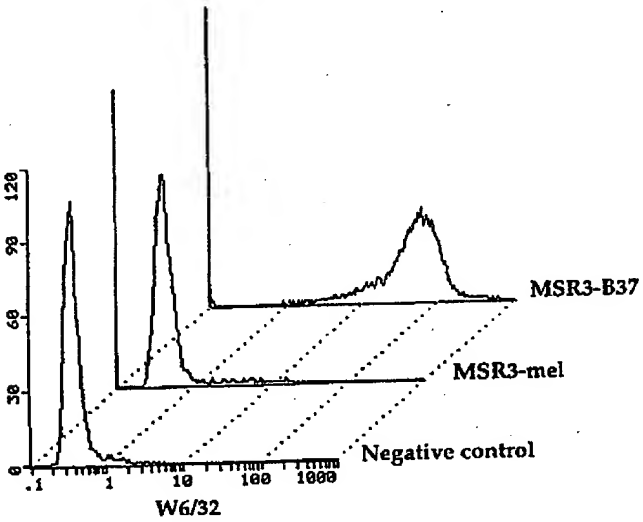

Fig. 1. Expression of HLA-class I molecules by MSR3-mel and MSR3-B37. Tumor cells were incubated with mAb W6/32 (anti-HLA-class I) or with an isotype control, washed, and labeled with goat antimouse immunoglobulin antibodies coupled to fluorescein. The analysis was performed before and after HLA-B*3701 transfection of MSR3-mel.

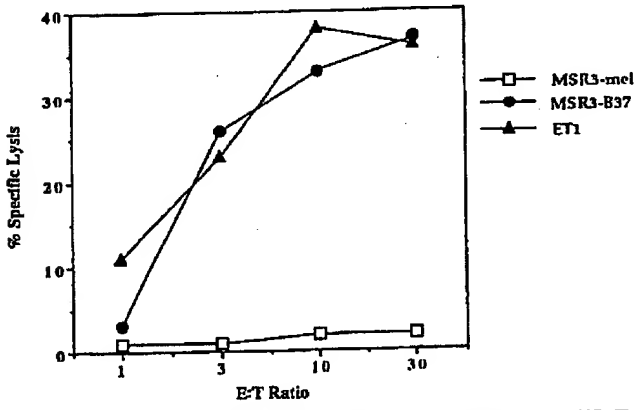

Fig. 2. Recognition of a shared HLA-B*3701-restricted antigen by CTL 337. The cytotoxic activity of CTL 337 cells was assessed against the autologous melanomas MSR3-mel and MSR3-B37 and against the allogenic melanoma ET1 at various E:T ratios.

the presence of *MAGE-4*- and *MAGE-12*-transfected Cos-7 cells (Fig. 3).

To identify the sequence coding for the antigenic peptide(s) recognized by CTL 337, we digested cDNA encoding *MAGE-1* with *Bgl*II and *Eco*RI obtaining two subfragments of ~495 and 1072 bp (Fig. 4). They were cloned into plasmid pcDNA 3.1 and transfected into Cos-7 cells along with HLA-B*3701 molecule. The presence of an in-frame start codon at 202 and 707 bp in the 495- and 1072-bp fragments, respectively, assured the expression of the two subfragments in the transfected cells. The level of IFN-γ released by CTL 337 cells in the presence of Cos-7 cells transfected with the 495-bp fragment was comparable to that conferred by the entire *MAGE-1* gene (Fig. 4), indicating that the antigenic peptide was encoded within this region. The amino acid sequence encoded by the 495-bp fragment (Fig. 5) was screened for peptides carrying the binding motif for HLA-B*3701 (31). Five peptides carrying aspartate or glutamate in position 2 and isoleucine or leucine in position 9/10 were identified (Fig. 5). One of these peptides, REPVTKAEML, was present also in the amino acid sequences encoded by *MAGE-2*, *MAGE-3*, and *MAGE-6*. This peptide, denominated MAGE.127–136, was used to sensitize the MSR3-EBV line to lysis by CTL 337 cells in a titration assay (Fig. 6*A*). The half-maximal lysis was reached with 90 nM peptide. No lysis of MSR3-EBV pulsed with an unrelated peptide that was able to bind to HLA-B*3701 was observed (Fig. 6*B* and data not shown).

Low levels of IFN-γ were released by the CTL 337 cells in the presence of Cos-7 cells expressing *MAGE-4* and *-12* (Fig. 3). To verify whether this release could be ascribed to recognition of peptides encoded by codons 127–136 within *MAGE-4* and *MAGE-12*, a peptide-binding study was performed, using MSR3-EBV cells pulsed with the two peptides as targets. Peptide $M4_{127-136}$, KELVTKAEML, differs by two amino acids (lysine *versus* arginine in position 1 and leucine *versus* proline in position 3) from peptide REPVTKAEML, whereas peptide $M12_{127-136}$, REPFTKAEML, differs by only one amino acid (phenyalanine *versus* valine in position 4). The results revealed that the two peptides can bind to HLA-B*3701 because increasing amounts of both were able to inhibit the lysis of MSR3-EBV pulsed with peptide REPVTKAEML but not with an unrelated HLA-A1-binding peptide (*i.e.*, M3.271–279; Fig. 6*B*). However, no recognition of EBV cells pulsed with peptides $M4_{127-136}$ and $M12_{127-136}$ was observed (data not shown).

Taken together, these data indicate that CTL 337 cells are able to recognize a peptide endogenously processed from *MAGE-1*, *-2*, *-3*, and *-6* products. The two peptides, encoded by the same region of *MAGE-4* and *-12*, respectively, are able to bind to HLA-B*3701, but they are not recognized by CTL 337 cells.

CTL 337 Cells Specifically Recognize *MAGE-2* and *-6* Gene Products. Until now, there was no evidence of the immunogenicity of *MAGE-2*- and *MAGE-6*-encoded proteins in humans. Indeed, peptides encoded by *MAGE-1*, *-3*, *-4*, and *-12* have been found to bind to various class I molecules to form antigens recognized by different CTLs, whereas no peptides encoded by the genes *MAGE-2* or *MAGE-6* have thus far been identified.

To demonstrate that peptide REPVTKAEML could also be processed from *MAGE-2* and *-6* and presented to CTL 337 cells, we attempted to look for melanoma cell lines expressing *MAGE-2* or *-6* but none of the other *MAGE* genes. Unfortunately, expression of the *MAGE* genes in melanomas is strictly correlated: most of the melanomas that express one member of the *MAGE* gene family also express the others. Indeed, we were unable to find a melanoma line

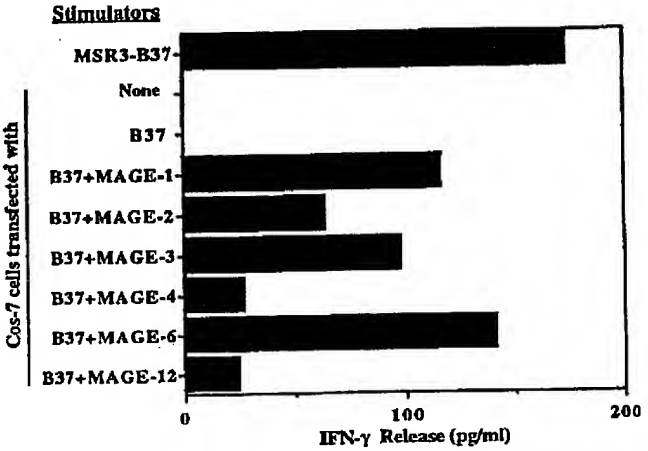

Fig. 3. Identification of the tumor-antigens recognized by CTL 337. Cos-7 cells were cotransfected with HLA-B*3701, alone or together with cDNAs encoding genes *MAGE-1*, *-2*, *-3*, *-6*, and *-12*. After 48 h, CTL 337 cells were added, and the IFN-γ released was measured 24 h later, as described in "Materials and Methods." MSR3-B37 was included as positive control.

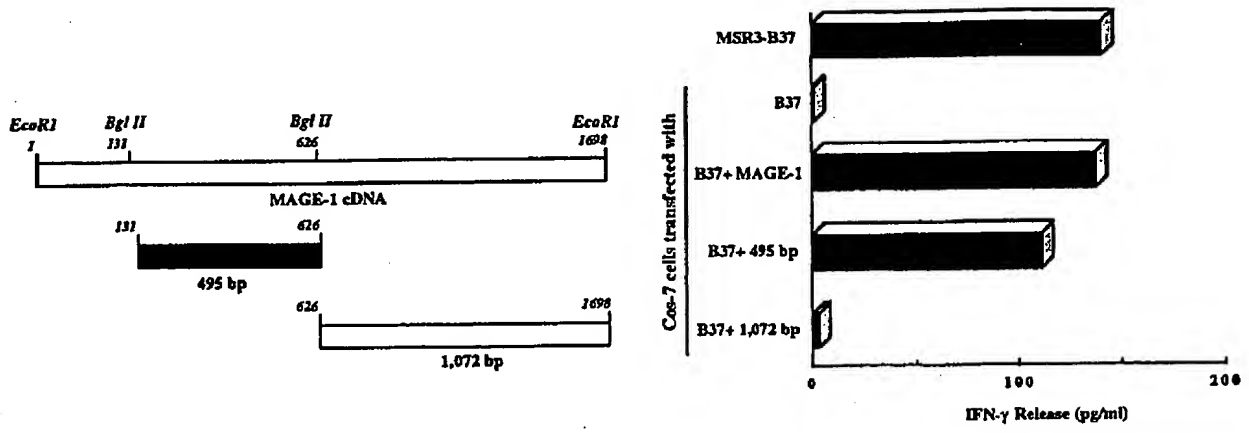

Fig. 4. Identification of the sequence coding for the antigenic peptide recognized by CTL 337. Cos-7 cells were cotransfected with two subfragments of *MAGE-1* cDNA together with HLA-B*3701. After 48 h, CTL 337 cells were added, and production of IFN-γ was measured 24 h later, as described in "Materials and Methods." As control, Cos-7 cells were transfected with HLA-B*3701, alone or together with the full-length *MAGE-1* cDNA.

that selectively expresses *MAGE-2*, but we succeeded in finding a single melanoma line, Me14932, that selectively expresses *MAGE-6* at low level (data not shown).

To verify whether peptide REPVTKAEML is endogenously processed from *MAGE-6* products and presented by HLA-B*3701, Me14932 was transduced by a retroviral vector encoding the HLA-B*3701 molecule. As indicated by immunofluorescence staining with a HLA-Bw4-specific mAb, cell surface expression of HLA-B*3701 on a pure population of transduced Me14932 cells was at least 2-fold lower than that of MSR3-B37 melanoma cells (data not shown). CTL 337 cells were able to recognize the Me14932-LB37 line in a cytotoxicity assay, and the level of lysis was increased by the exogenous addition of peptide REPVTKAEML, whereas there was no recognition of the pulsed and unpulsed Me14932 lines (Fig. 7). The low levels of lysis of the melanoma Me14932-LB37 might be explained either by weak expression of gene *MAGE-6* and by the weak surface expression of HLA-B*3701 molecules.

To evaluate whether the inclusion of *MAGE-2* and *-6* in the list of possible target antigens for specific immunotherapy could increase the proportion of eligible patients, we analyzed the expression of *MAGE-1*, *-2*, *-3*, and *-6* in fresh tumor samples of various histotypes. Melanomas were not analyzed because expression of the different *MAGE* genes was clearly correlated (32). The results indicate that 12% of the ovarian carcinomas and 5% of colon and breast carcinomas express *MAGE-2* and/or *-6* in the absence of *MAGE-1* and *-3* (Table 1). On the other hand, in all bladder and lung carcinomas studied the four genes were always coexpressed.

In conclusion, the data reported in this study indicate that *MAGE-2* and *-6* can be included in the list of possible target antigens for

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gaagatctgc ctgtgggtct tcattgccca gctcctgccc acactcctgc ctgctgccct gacgagagtc atcatgtctc ttgagcagag 221

                                L  E  A  Q  Q  E  A  L  G  L
gagtctgcac tgcaagcctg aggaagccct tgaggcccaa caagaggccc tgggcctggt gtgtgtgcag gctgccacct cctcctcctc 311

tcctctggtc ctgggcaccc tggaggaggt gcccactgct gggtcaacag atcctcccca gagtcctcag ggagcctccg cctttcccac 401

                                                                E  E  G  P  S  T  S  C  I / L  E  S
taccatcaac ttcactcgac agaggcaacc cagtgagggt tccagcagcc gtgaagagga ggggccaagc acctcttgta tcctggagtc 491

 L  F  R  A  V  I                 A  D  L  V  G  F  L  L  L                    R  E  P  V  T  K  A
cttgttccga gcagtaatca ctaagaaggt ggctgatttg gttggttttc tgctcctcaa atatcgagcc agggagccag tcacaaaggc 581

 E  M  L
agaaatgctg gagagtgtca tcaaaaatta caagcactgt tttcc                                              671
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Mage-1	REPVTKAEML
Mage-2	REPVTKAEML
Mage-3	REPVTKAEML
Mage-4	KELVTKAEML
Mage-6	REPVTKAEML
Mage-12	REPFTKAEML

Fig. 5. *Top*, sequence of the 495-bp subfragment of *MAGE-1* cDNA. Peptides carrying the binding motifs for HLA-B*3701 are listed above their respective nucleotide sequences. *Bottom*, comparison of peptide REPVTKAEML (*underlined*), encoded by *MAGE-1*, with the peptides encoded by the homologous regions of other genes of the *MAGE* family.

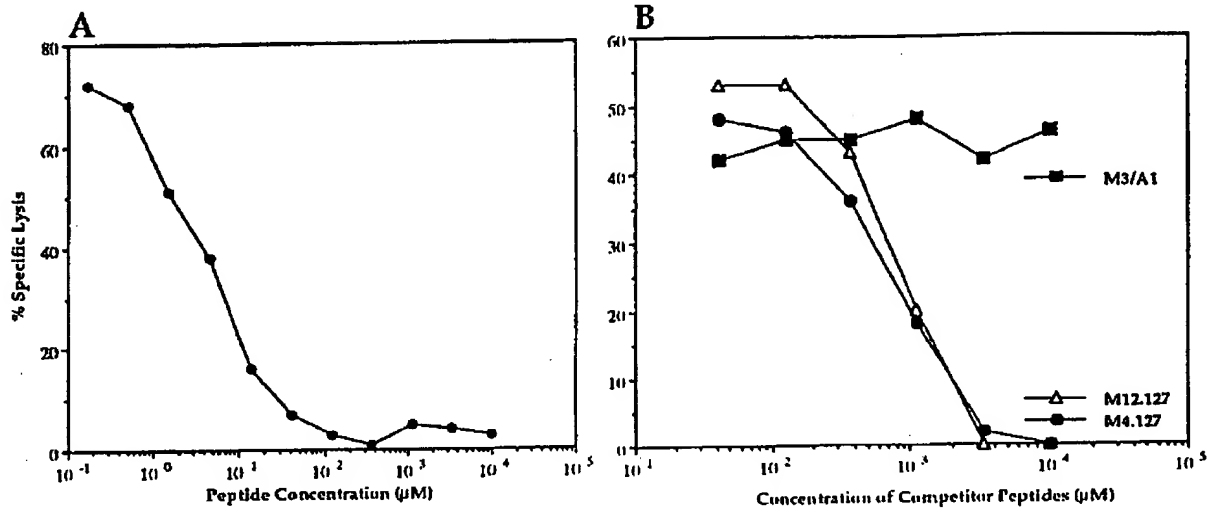

Fig. 6. *A*, recognition of the peptide $MAGE_{127-136}$ by CTL 337 cells. MSR3-EBV cells were incubated with 3-fold dilutions of peptide $MAGE_{127-136}$, starting from 10 mM and used as target cells in a standard cytotoxicity assay. The E:T ratio was fixed at 10:1. *B*, binding of peptides $M4_{127-136}$ and $M12_{127-136}$ to HLA-B*3701, evaluated in a competition assay. Competitor peptides included the $M4_{127-136}$ peptide KELVTKAEML and the $M12_{127-136}$ peptide REPFTKAEML. The M3.A1 (*i.e.*, $M3_{271-279}$) peptide, which was unable to bind to the HLA-B*3701 molecule, was used as negative control. Percentage lysis without competitor peptides was 52%.

tumor-specific immunotherapy, increasing the number of patients that could benefit from this therapy.

DISCUSSION

In the last few years, there has been a considerable effort to characterize antigenic peptides encoded by tumor-associated antigens and the HLA molecules responsible for their presentation (6). Several immunotherapy clinical trials of cancer vaccinations based on the use of these peptides are in progress, with quite positive preliminary results. Indeed, some objective cancer responses have been observed, consisting of both tumor regression (3, 5) and a few long-term complete responses (4).

One major limitation outlined by those studies is the development, in a significant proportion of the treated patients, of tumor variants that fail to express the antigen recognized by tumor-reactive lymphocytes (24). Those variants can be generated either by loss of the nominal antigen (referred to as antigen loss variants; Refs. 33 and 34) or by molecular defects affecting different steps

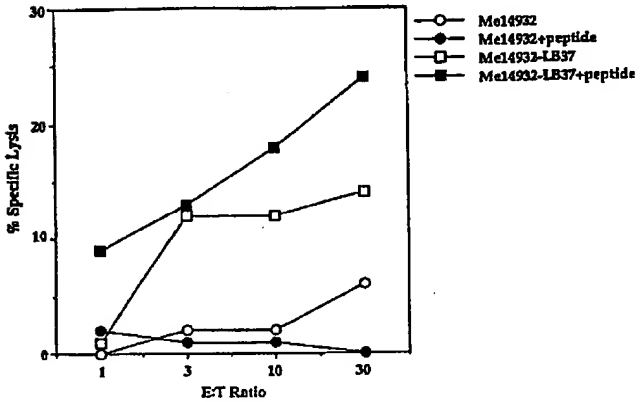

Fig. 7. Recognition of a *MAGE*-6-positive melanoma cell line by CTL 337. The HLA-B*3701-negative line Me14932 and the HLA-B*3701-positive line Me14932-LB37 were pulsed or not with 16 μM peptide $MAGE_{127-136}$ and used as target cells in a standard cytotoxicity assay at the indicated E:T ratios.

Table 1 *Expression of MAGE genes by fresh tumor samples*[a]

Histological type	% RT-PCR-positive tumors				
	MAGE-1	*MAGE-3*	*MAGE-2*	*MAGE-6*	*MAGE-2* or *MAGE-6* only
Lung carcinoma (28)[b]	35	39	32	29	0
Breast carcinoma (20)	30	10	10	15	5
Ovary carcinoma (25)	24	20	32	20	12
Bladder carcinoma (25)	28	28	20	24	0
Colon carcinoma (17)	0	5	5	5	5

[a] As determined by reverse transcriptase-PCR (RT-PCR) analysis.
[b] Numbers in parentheses represent numbers of fresh tumor samples analyzed.

of the antigen presentation pathway (referred to as presentation loss variants; Refs. 35–37). An active intervention of the immune system in the selection of antigen loss and presentation loss variants have been observed in both treated (24) and untreated patients (33, 38). However, escape from classical tumor-specific CTLs may be counteracted *in vivo* by the intervention of different immune effectors. Indeed, tumor cells that have lost expression of some but not all HLA class I molecules can be recognized by a new category of antitumor lymphocytes expressing killer-cell inhibitory receptors (39), whereas HLA-negative tumor cells can be targeted by NK cells.

The melanoma cell line used in this study belongs to the presentation loss variant class of HLA-negative tumor cells. The molecular defect responsible for the HLA class I phenotypes of MSR3-mel has not yet been identified; however, our melanoma line exhibits barely detectable levels of HLA class I expression by immunofluorescence analysis, which are not sufficient for stimulation of a tumor-specific T-cell response (data not shown). This altered phenotype does not seem to be due to β-2m or TAP alterations or to deletions of MHC genes but rather to a defect in the transcriptional machinery. Indeed, HLA class I expression in MSR3-mel can be restored by transfection of cDNAs encoding autologous HLA class I alleles.

The HLA-B*3701-transfected cell line (*i.e.*, MSR3-B37) allowed the isolation of HLA-B*3701-restricted and tumor-specific CTLs that recognized a nonapeptide encoded by the same region (*i.e.*, residues 127–136) of MAGE-1, -2, -3, and -6 proteins.

To our knowledge, this is the first B*3701-restricted tumor-specific epitope that has been identified thus far. Note that the HLA-B*3701 molecule is present on both lymphocytes and tumor cells of patient MZ2 (7), from which a large variety of *MAGE*-specific CTL clones restricted by different HLA class I molecules were isolated (8–9, 40–42). Those results suggest a subdominant role of HLA-B*3701 in tumor antigen presentation in the MZ2 model that should be overcome in the MSR3 system by the absence on the stimulating cells of a HLA class I molecule other than HLA-B*3701. Indeed, dominance of a given HLA molecule in the tumor-specific stimulation of autologous CTL by melanoma cells has been described in several model systems (39, 43, 44).

Several members of the MAGE gene family are specifically expressed by tumors of various histological types and T-cell defined epitopes encoded by *MAGE-1* and *-3* have been identified. However, although *MAGE-2* and *-6* are expressed in a large percentage of tumor samples, thus far no *MAGE-2*- and *MAGE-6*-specific CTLs have been isolated. The only suggestion that *MAGE-2* behaves like a tumor-antigen comes from the study of Visseren *et al.* (45), who demonstrated the immunogenicity of *MAGE-2* in a HLA-A*0201Kb transgenic mouse model. Therefore, our study reports the first evidence for an immunogenic potential of *MAGE-2* and *-6* in humans. Indeed, CTL 337 cells were able to recognize Cos-7 cells transfected with HLA-B*3701 and *MAGE-2* or *-6* genes. Moreover, a stable HLA-B*3701-positive melanoma line expressing *MAGE-6* was recognized, whereas analogous experiments on *MAGE-2* and HLA-B*3701-positive melanoma lines could not be performed. It has been suggested that the proteasome specifically digests proteins into polypeptides with defined hydrophobic, basic, or acidic COOH termini, whereas the NH_2-terminal cleavage into smaller fragments occurs nonspecifically 8–10 amino acids further upstream. In view of the presence of hydrophobic residues (M and L) at the COOH-terminus of peptide REPVTKAEML, as well as the high degree of the amino acid sequence homology between *MAGE-2* and *MAGE-6* in the region around peptide REPVTKAEML, it is tempting to speculate that this peptide might indeed be processed in melanoma cells also from *MAGE-2* products. (46, 47).

The molecular analysis performed on tumor samples of various histotypes revealed a strong correlation between the expression of different *MAGE* genes. However, inclusion of *MAGE-2* and *MAGE-6* in the list of target antigens for cancer immunotherapy has practical implications for the enrollment of patients with ovarian carcinomas. Indeed, 12% of the ovarian carcinoma samples analyzed express *MAGE-2* and/or *-6*, without expressing *MAGE-1* and/or *-3* genes. On the other side, coexpression of more than one *MAGE* gene by a given tumor might prevent the development of antigen loss variants during vaccination treatment. Indeed, immune escape from a peptide-induced antitumor response might then be rare, because it would require the occurrence of several independent molecular alterations.

In conclusion, the identification of this new HLA-B*3701-restricted epitope not only increases the number of patients eligible for immunization but also may prove highly efficient for immunotherapy because of reduced risk of tumor escape due to the emergence of antigen loss variants.

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Dis ase-associated Bias in T Helper Type 1 (Th1)/Th2 CD4$^+$ T Cell Responses Against MAGE-6 in HLA-DRB1*0401$^+$ Patients With Renal Cell Carcinoma or Melanoma

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Abstract

T helper type 1 (Th1)-type CD4$^+$ antitumor T cell help appears critical to the induction and maintenance of antitumor cytotoxic T lymphocyte (CTL) responses in vivo. In contrast, Th2- or Th3/Tr-type CD4$^+$ T cell responses may subvert Th1-type cell-mediated immunity, providing a microenvironment conducive to disease progression. We have recently identified helper T cell epitopes derived from the MAGE-6 gene product; a tumor-associated antigen expressed by most melanomas and renal cell carcinomas. In this study, we have assessed whether peripheral blood CD4$^+$ T cells from human histocompatibility leukocyte antigens (HLA)-DRβ1*0401$^+$ patients are Th1- or Th2-biased to MAGE-6 epitopes using interferon (IFN)-γ and interleukin (IL)-5 enzyme-linked immunospot assays, respectively. Strikingly, the vast majority of patients with active disease were highly-skewed toward Th2-type responses against MAGE-6–derived epitopes, regardless of their stage (stage I versus IV) of disease, but retained Th1-type responses against Epstein-Barr virus– or influenza-derived epitopes. In marked contrast, normal donors and cancer patients with no current evidence of disease tended to exhibit either mixed Th1/Th2 or strongly Th1-polarized responses to MAGE-6 peptides, respectively. CD4$^+$ T cell secretion of IL-10 and transforming growth factor (TGF)-β1 against MAGE-6 peptides was not observed, suggesting that specific Th3/Tr-type CD4$^+$ subsets were not common events in these patients. Our data suggest that immunotherapeutic approaches will likely have to overcome or complement systemic Th2-dominated, tumor-reactive CD4$^+$ T cell responses to provide optimal clinical benefit.

Key words: melanoma • renal cell carcinoma • helper T lymphocyte • MAGE-6 • epitope

Introduction

Although renal cell carcinoma (RCC)* and melanoma are considered among the most responsive cancers to immunotherapy, the vast majority of recent immunotherapeutic approaches have focused solely on the induction of CD8$^+$ antitumor T cells in vivo as a surrogate of clinical benefit (1–3). It appears clear that the ability to promote effector CD8$^+$ T cells reactive against tumor antigens is a necessary, but not sufficient, event in objective clinical responses (4). CD4$^+$ T cells can also recognize tumor antigen-derived peptides, either directly (as some RCCs and melanomas express MHC class II molecules in situ; references 5 and 6) or via cross-presentation mechanisms by host antigen-presenting cells, such as dendritic cells (DCs; references 7 and

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Abbreviations used in this paper: DC, dendritic cell; IVS, in vitro stimulation(s); RCC, renal cell carcinoma.

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8). Th1-type $CD4^+$ T cells secreting IFN-γ appear crucial to the optimal generation and durability of specific CTL in vivo and may also serve to recruit these effector cells into the tumor microenvironment via delayed-type hypersensitivity responses (8). Hence, the lack of effectively promoting specific Th1-type $CD4^+$ T cell generation, maintenance, and direction for antitumor $CD8^+$ T cells may represent a significant limitation in current vaccine trials.

Tumor-induced deviation of $CD4^+$ T cell responses in progressive disease and the role of Th1- and Th2-type $CD4^+$ effector cells have been evaluated in a limited number of murine models (9–19). Studies using the B16 melanoma model have documented a gradual shift of initial Th0-, mixed Th1-/Th2-type $CD4^+$ T cell response to Th2/Tr-type dominated responses by 14–20 d of progressive tumor growth (13, 17–19). Injection of neutralizing anti–IL-4, -IL-10, or -TGF-β1 antibodies can prevent this tumor-induced functional transition, resulting in enhanced $CD8^+$ CTL generation and protection against tumor growth (17). Depletion of $CD4^+$ T cells in late-stage progressive B16 models, where Th2/Tr-type response dominate, restores CTL effector function and can result in tumor regression and vitiligo, particularly upon administration of rIL-12 (13). Analyses of the anti-tumor efficacy of Th1- and Th2-type $CD4^+$ T cells has also been evaluated in prophylactic and adoptive transfer tumor models (9, 12, 13, 15). In these latter cases, Th1- and Th2-type can mediate complementary antitumor effector functions, via contrasting mechanisms (9). Although Th2-type $CD4^+$ T cells can promote the recruitment of tumoricidal eosinophils and macrophages into the tumor microenvironment and promote acute tumor rejection (9), on a cell-per-cell basis Th1-type T cells appear to provide a greater therapeutic index (12, 14, 15) and only Th1-type $CD4^+$ T cells appear to promote durable anti-tumor CTL responses (15).

Interestingly, tumor infiltrating lymphocytes in patients with spontaneous and therapeutically induced regressing lesions appear to be characterized by dominant Th1-type responses to mitogens, whereas tumor infiltrating lymphocytes from patients with progressor lesions have been reported to exhibit functionally dominant Th2-type (IL-4, IL-5) and/or Th3-/Tr-type (IL-10, TGF-β1) $CD4^+$ T cell responses (20–22). However, prior analyses of patient bulk $CD4^+$ T cell responses to mitogenic stimuli have yielded equivocal results, with generally no clear-cut Th1- or Th2-type bias observed (23–25). As this difference in results may reflect the stringency with which tumor-specific $CD4^+$ T cell responses were evaluated, significant recent emphasis has been placed on the identification of tumor antigen-derived helper T cell epitopes that may be used to quantitate and assess tumor-specific $CD4^+$ T cell numbers and function.

Based on IFN-γ as a read-out cytokine or proliferation as an endpoint, Th1-type epitopes have been identified for the MART-1/Melan-A, gp100/pmel17, tyrosinase, MAGE-3, and MAGE-6 (unpublished data) melanoma-associated antigens, among others (26–28). We have recently defined a set of helper epitopes derived from the MAGE-6 protein and have focused on these targets in the current study, since expression of MAGE-6 (unpublished data) has been observed in premalignant lesions in situ and at high frequencies in primary and metastatic tumors (29–31). Hence, $CD4^+$ T cell responses to MAGE-6 epitopes may represent early etiologic events. The bias of MAGE-6 (tumor)-specific $CD4^+$ T cell responses (i.e., Th1, Th2, Th3/Tr) may impact cancer incidence in susceptible individuals, the progression status of established MAGE-6^+ tumors or time to recurrence of MAGE-6^+ disease in the adjuvant setting. Indeed, melanoma expression of HLA-DR has been reported to be a marker of poor prognosis (32, 33), suggesting that the nature of CD4+ T cell recognition of MHC class II–presented tumor epitopes may play a decisive immunoregulatory role in situ.

This study is the first to demonstrate tumor antigen-specific Th2-type polarization of $CD4^+$ T cell responses in the peripheral blood of patients with RCC or melanoma. We have implemented IFN-γ and IL-5 ELISPOT assays to assess the magnitude of Th1- and Th2-type $CD4^+$ T cell responses to MAGE-6 epitopes, respectively. We report that HLA-DRβ1*0401^+ patients with active melanoma or RCC displayed strongly polarized Th2-type reactivity to these peptides, whereas normal donors and patients that were disease-free following therapeutic intervention exhibited either weak mixed Th1-/Th2-type or strongly-polarized Th1-type responses to these same epitopes.

Materials and Methods

Cell Lines and Media. The T2.DR4 (DRB1*0401^+) cell line (provided by Dr. Janice Blum, Indiana University School of Medicine, Indianapolis, IN) was used as the peptide-presenting cell in these studies. This cell line uniformly expresses HLA-DR*0401 molecules that contain moderate-to-low affinity binding peptides derived mainly from intracellular invariant chain (class II–associated invariant chain peptide [CLIP]) due to a genetic deficiency in HLA-DM (34). T2.DR4 cells were maintained in RPMI-1640 supplemented with 10% heat-inactivated FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10 mM L-glutamine (all reagents GIBCO BRL).

Peptide Selection and Synthesis. The MAGE-6 (unpublished data), influenza A $matrix_{60-73}$ (35), malarial $circumsporozoite_{326-345}$ (26) and EBV $EBNA\text{-}1_{519-533}$ (36) HLA-DR4-presented epitopes were synthesized by FMOC chemistry by the University of Pittsburgh Cancer Institute's (UPCI) Peptide Synthesis Facility (Shared Resource). Peptides were >90% pure based on HPLC profile and MS/MS mass spectrometric analysis performed by the UPCI Protein Sequencing Facility (Shared Resource).

Isolation of Patient and Normal Donor PBMC-Derived T Cells. 40–100 ml of patient or normal donor heparinized blood was obtained with informed consent under IRB-approved protocols and diluted 1:2 with HBSS, applied to ficoll-hypaque gradients (LSM; Organon-Teknika) per the manufacturer's instructions, and centrifuged at 550 *g* for 25 min at room temperature. Patient and normal donor information is provided in Table I. PBMCs at the buoyant interface were recovered and washed twice with HBSS to remove residual platelets and ficoll-hypaque. HLA-$DR4^+$ status was confirmed by flow cytometry using the anti-HLA-DR4

Table I. *HLA-DRB1*0401-positive Patients Evaluated in this Study*

Patient	Age	Sex	Stage	Treatment	Disease status at time of evaluation (y)	RT-PCR MAGE-6 (+/−)	α-MAGE response 121	140	246
Melanoma (n = 18)									
SLM1	71	F	IV	S, DC/peptide	NED (1.5)	+	Th1	Th1	Th1
SLM2	64	F	IV	S, IFN-α	NED (5.0)	+	Th1	Th1	Th1
SLM5	34	M	IV	S, IFN-α	NED (4.1)	NA	Th1	Th1	−
SLM6	37	F	I	S, IFN-α	NED (1.9)	NA	Th1	Th1	Th1
SLM9	35	F	III	S, C, R	NED (0.3)	+	Th1	−	−
SLM10	74	M	IV	S	NED (0.4)	NA	Th1	−	−
SLM12[a]	39	F	IV	S, C, IFN-α	Stable	+	Th2	Th2	Th2
SLM14	52	M	IV	C	Mets, brain	+	Th2	Th2	Th2
SLM16	64	M	IV	None	Mets, liver/lung	+	−	−	Th2
SLM17	74	M	IV	S	Mets	+	Th2	Th2	Th2
SLM18	31	F	IV	S, IFN-α	Mets, brain	+	Th2	Th2	Th2
SLM19	56	M	IV	S, IFN-α, C	Mets	+	−	Th2	Th2
SLM21	45	M	IV	S, IFN-α	Mets	−	−	−	−
SLM22	57	F	IV	S	Mets	−	Th2	Th2	Th2
SLM23	63	M	I	S, IFN-α	NED (1.2)	+	Th2	Th1/2	−
SLM24	36	F	IV	S	Mets	+	Th2	Th2	Th2
SLM25	42	M	IV	S	Mets	+	−	Th2	−
SLM26	41	F	IV	S	Mets	−	−	−	−
RCC (n = 18)									
SLR2	51	F	IV	S	NED (0.3)	+	Th1	Th1	Th1
SLR3	45	M	IV	S	Mets	NA	−	Th2	−
SLR4	49	F	IV	S, IL-2	Mets	NA	Th2	Th2	Th2
SLR5	79	M	IV	S, IFN-α	Mets	NA	Th2	Th1/2	Th2
SLR6	64	M	I	S	NED (0.3)	NA	Th1	Th1	Th1
SLR7	52	F	I	S	Local Dis.	NA	−	−	Th2
SLR8	49	M	IV	C, R	Mets	NA	−	Th2	−
SLR9	53	F	I	S	NED (0.1)	NA	Th1	−	−
SLR10	41	M	IV	S, C, R	Mets	NA	Th2	−	−
SLR11	58	M	IV	S, IFN-α, R	Mets	−	−	−	−
SLR12	58	M	I	S	Local Dis.	NA	−	−	Th2
SLR13	71	M	I	S	Local Dis.	NA	−	−	−
SLR14	75	F	I	S	Local Dis.	NA	−	Th2	−
SLR15	58	M	I	S	NED (0.1)	NA	Th1	−	−
SLR16	57	M	IV	S, R	Mets	−	−	−	Th2
SLR17	53	M	II	S	Local Dis.	NA	−	−	Th2
SLR18	62	F	II	S	Local Dis.	NA	Th2	Th2	Th2
SLR19	67	M	IV	S, IFN-α, R	Mets	NA	Th2	Th2	Th2

AIT, adoptive immunotherapy (VDLN cells); C, chemotherapy; Mets, metastatic disease; R, radiotherapy; S, surgery; DC/peptide, dendritic cell plus synthetic melanoma peptide vaccine; IFN-α, IFN-α therapy; NED, no evidence of disease at time of blood draw; NA, not available for evaluation. [a]Patient with ocular melanoma. Th1 or Th2 assignment for peptide reactivity reflects donor responses of ≥10 spots/50,000 CD41 T cells as determined in IFN-γ or IL-5 ELISPOT assays, respectively.

reactive mAb clone 359–13F10 (IgG; provided by Dr. Janice Blum, Indiana University School of Medicine, Indianapolis, IN) in indirect immunofluorescence assays. PBMCs were diluted to 10^7/ml in AIM-V medium (GIBCO BRL) and incubated for 60 min at 37°C in T75 vented flasks (COSTAR), with subsequently harvested adherent cells used to generate DCs (see below) and nonadherent cells frozen in 90% FCS containing 10% DMSO (Sigma-Aldrich) at 10^7 lymphocytes/vial using controlled-rate freezing technique. On the day of establishing DC–T cell cultures, nonadherent cells were thawed and washed twice with HBSS. $CD4^+$ T cells were then isolated using MACS™ (Miltenyi Biotec) anti–human CD4 beads and MiniMACS™ columns per the manufacturer's protocol. $CD4^+$ T cell yields were typically 25–35% of starting PBMC numbers loaded, with purity exceeding 97% as assessed by flow cytometry.

Induction of Antitumor T Effector Lymphocytes. Autologous DCs were prepared as described previously in 7-d cultures of plastic-adherent PBMCs in AIM-V media supplemented with rhGM-CSF and rhIL-4 (26). Harvested, nonadherent DC (2×10^5) were then cocultured with 2×10^6 autologous $CD4^+$ T cells in the presence of 10 μM synthetic peptides for 7 d in RPMI-1640 containing 10% FBS and no exogenously added cytokines. Responder T cells were then harvested and analyzed for MAGE-6 peptide specificity in ELISPOT assays.

IFN-γ and IL-5 ELISPOT Assays for Peptide-Reactive $CD4^+$ T Cell Responses. To evaluate the frequencies of peripheral blood $CD4^+$ T cells recognizing peptide epitopes, ELISPOT assays for IFN-γ and IL-5 were performed as described previously (26, 27, 37). Briefly, $CD4^+$ T cell responses were evaluated by both IFN-γ (Th1) and IL-5 (Th2) ELISPOT assays. For ELISPOT assays, 96-well multiscreen hemagglutinin antigen plates (Millipore) were coated with 10 μg/ml of anti–human IFN-γ mAb (1-D1K; Mabtech) or 5 μg/ml of anti–human IL-5 (BD Biosciences) in PBS (GIBCO BRL/Life Technologies) overnight at 4°C. Unbound antibody was removed by four successive washing with PBS. After blocking the plates with RPMI1640/10% human serum (1 h at 37°C), 10^5 $CD4^+$ T cells and T2.DR4 cells (2×10^4 cells) were seeded in multiscreen hemagglutinin antigen plates. Synthetic peptides (stocks at 1 mg/ml PBS) were then added to appropriate wells at a final concentration of 10 μg/ml. Negative peptide control wells contained $CD4^+$ T cells with T2.DR4 cells pulsed with Malaria-$CS_{326-345}$ peptide, with T2.DR4 cells alone serving as the APC control. Positive controls were T cells plated in the presence of 5 μg/ml PHA (Sigma-Aldrich). Culture medium was AIM-V (GIBCO BRL/Life Technologies) at a final volume of 200 μl/well. Plates were incubated at 37°C in 5% CO_2 for 24 h in the case of IFN-γ ELISPOT assays and for 40 h in IL-5 ELISPOT assays. After incubation, supernatants of culture wells were harvested for ELISA analysis, and plates washed with PBS/0.05% Tween 20 (PBS/T) to remove cells. Captured cytokine was detected at sites of its secretion by incubation for 2 h with biotinylated mAb anti–human IFN-γ (7-B6–1; Mabtech) at 2 μg/ml in PBS/0.5% BSA or biotinylated mAb anti–human IL-5 (BD Biosciences) at 2 μg/ml in PBS/0.5% BSA. Plates were then washed six times with PBS/T, and avidin-peroxidase complex (diluted 1:100; Vectastain Elite Kit; Vector Laboratories) was added for 1 h. Unbound complex was removed by three successive washings with PBS/T and three rinses with PBS alone. AEC substrate (Sigma-Aldrich) was added and incubated for 5 min for the IFN-γ ELISPOT assay and the TMB substrate for peroxidase (Vector Laboratories) was added and incubated for 10 min for the IL-5 ELISPOT assay. All determinations were performed in triplicates, with spots imaged using the Zeiss AutoImager (and statistical comparisons determined using a Student two-tailed *t* test analysis). The data are represented as mean IFN-γ or IL-5 spots per 100,000 $CD4^+$ T cells analyzed.

TGF-β1 and IL-10 ELISAs. Supernatants were harvested from ELISPOT plates at the endpoint of the culture period and pooled for a single stimulus (i.e., a given peptide, etc.) and frozen at –20°C until analysis by cytokine-specific ELISA. Cytokine capture and detection antibodies and recombinant cytokine were purchased from BD Biosciences and used in ELISA assays per the manufacturer's instructions. The lower limit of detection for the TGF-β assay was 60 pg/ml, while that of the IL-10 ELISA was 7 pg/ml. In the case of IL-10, 1–5 responder $CD4^+$ T cells spots imaged in an IL-10 ELISPOT equated with ~12–17 pg/ml IL-10 as determined in the IL-10 ELISA assay (unpublished data).

PCR Analysis. PCR analyses were performed to determine patient HLA-DR4 genotype using a commercial PCR panel according to the manufacturer's instructions (Dynal) and PBL. RT-PCR analysis was also used to determine tumor expression of MAGE-6 mRNA. The following primer set was used: MAGE-6 (forward: TGGAGGACCAGAGGCCCCC, reverse: CAGGATGATTATCAGGAAGCCTGT, product size 728 bp with cycles: melting 94°C for 1 min, annealing 68°C for 1 min, extension 72°C for 1 min).

Results

*Normal HLA-DRβ1*0401$^+$ Donors Fail to React or Display a Mixed Th1/Th2 $CD4^+$ Response to MAGE-6 Epitopes after Primary In Vitro Stimulation.* MAGE-6 peptides were not recognized by freshly-isolated $CD4^+$ T cells harvested from normal HLA-DRB1*0401$^+$ donors (unpublished data). To determine if $CD4^+$ precursors were present in normal donors and to identify the balance of Th1-type versus Th2-type responses, we implemented IFN-γ and IL-5 ELISPOT assays, respectively. Although both IL-4 and IL-5 have been previously reported as signature cytokines for Th2-type T cell responses (38, 39), we chose the IL-5 assay over the IL-4 ELISPOT assay to screen for Th2-type $CD4^+$ T cell reactivity for technical reasons, mainly the much lower backgrounds and higher signal-to-noise ratio observed for the IL-5 ELISPOT system (37, 40).

To evaluate whether normal donors could be prompted to recognize any of these sequences, isolated $CD4^+$ T cells derived from the PBMCs of HLA-$DR4^+$ normal donors were stimulated with autologous immature DCs in the presence of the individual MAGE-6 peptides (i.e., MAGE-$6_{121-144}$, MAGE-$6_{140-170}$, or MAGE-$6_{246-263}$). We chose this in vitro stimulation (IVS) protocol since the DCs generated were poor IL-12 producers upon CD40 ligation and these antigen presenting cells do not appear to skew $CD4^+$ T cell responses in either a Th1-type or Th2-type manner (unpublished data). Hence, we consider these as "neutral" DC for $CD4^+$ T cell stimulations. 1 wk after stimulation, $CD4^+$ T cells were used as responders against T2.DR4 target cells pulsed with the candidate DR4-binding peptides. As shown in Fig. 1, an analysis of 10 normal HLA-$DR4^+$ donors revealed either very low responsiveness or responses that were equivocal with regard to their balance of IFN-γ versus IL-5 spot production. Although

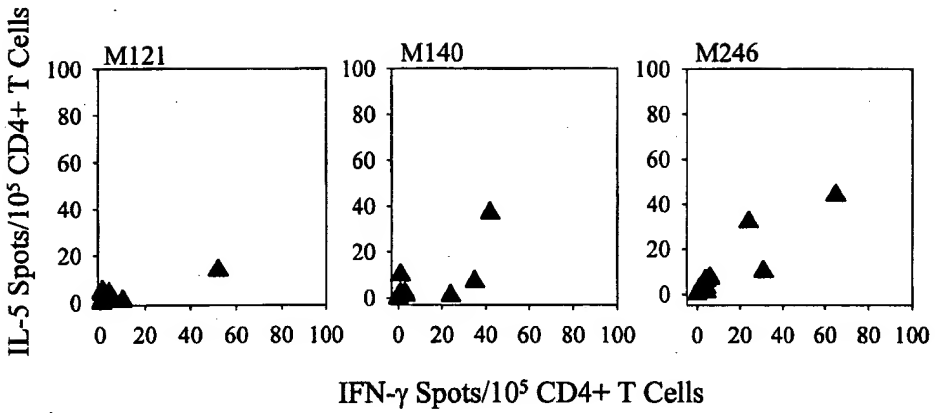

Figure 1. Analysis of the Th1-type vs. Th2-type $CD4^+$ T cell response to MAGE-6 peptides in HLA-DRβ1*0401$^+$ normal donors. Peripheral blood $CD4^+$ T cells were isolated from normal donors and stimulated with autologous, "immature" DCs in the presence of the $MAGE\text{-}6_{121\text{–}144}$ (M121), $MAGE\text{-}6_{140\text{–}170\ (140L)}$ (M140), or $MAGE\text{-}6_{246\text{–}263}$ (M246) peptides for 7 d, in the absence of exogenous cytokines. Responder $CD4^+$ T cells were then analyzed for their reactivity to T2.DR4 cells pulsed with individual MAGE-6 peptides in both IFN-γ and IL-5 ELISPOT assays. Each symbol within a panel represents the combined IFN-γ/IL-5 ELISPOT data for an individual normal donor.

we did not perform these analyses in a manner that allowed for an assessment of coordinate cytokine production from a given $CD4^+$ T cell, we considered these results from normal donors to reflect Th0-type or mixed Th1-type/Th2-type responses.

Th1-type versus Th2-type Immunoreactivity of $CD4^+$ T Cells Against HLA-DR4-Presented MAGE-6 Epitopes in HLA-DR4$^+$ RCC or Melanoma Patients. Recent reports have suggested that $CD4^+$ T cells infiltrating progressor RCC and melanoma may display a predominant Th2-bias in response to TCR ligation (41, 42). We used the IFN-γ (Th1-type) and IL-5 (Th2-type) ELISPOT assays to discern whether such a bias existed in the peripheral blood $CD4^+$ T cell repertoire of HLA-DRβ1*0401$^+$ patients with RCC or melanoma using the identical IVS system outlined above for normal donors.

Overall, the peripheral blood $CD4^+$ T cell responses were evaluated from 18 RCC and 18 melanoma patients (Table I). Among these patients, 4/18 RCC patients and 7/18 melanoma patients were disease-free (i.e., no-evidence of disease [NED]) at the time of analysis, with all other patients presenting with active disease. As shown in Fig. 2, patients with active disease (either RCC or melanoma) displayed strongly Th2-polarized $CD4^+$ T cell responses, whereas patients that were disease-free at the time of analysis were strongly Th1-polarized in their reactivity to the three MAGE-6 epitopes evaluated. Not every patient reacted against each of the peptides tested, but if they did respond to a given epitope, this response was strongly polarized in accordance with the disease status of the individual (i.e., Th2 if active disease, etc., Table I). Whereas the melanoma patients were all essentially diagnosed with

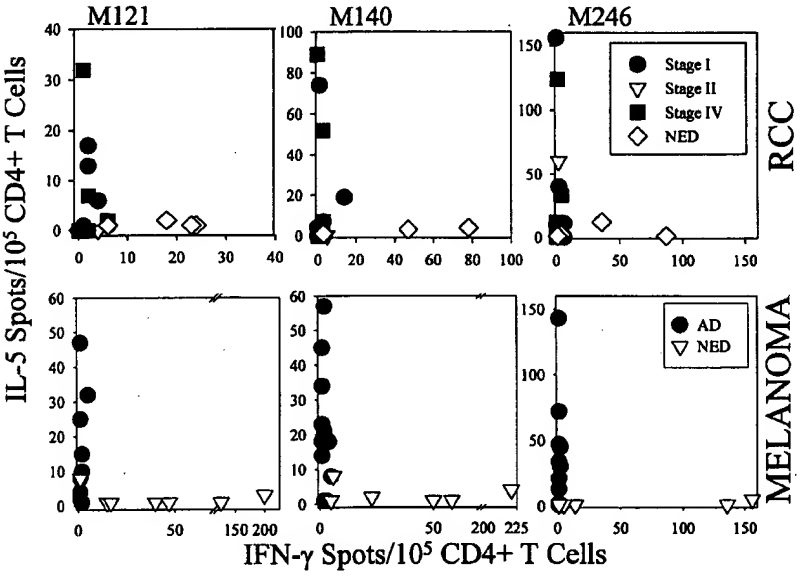

Figure 2. Analysis of the Th1-type vs. Th2-type $CD4^+$ T cell response to MAGE-6 peptides in HLA-DRβ1*0401$^+$ patients with RCC or melanoma. Peripheral blood $CD4^+$ T cells were isolated from patients with RCC or melanoma and stimulated for 7 d with autologous "immature" plus MAGE-6 peptides as described in the legend to Fig. 1. Responder $CD4^+$ T cells were then analyzed for their reactivity to T2.DR4 cells pulsed with individual MAGE-6 peptides in both IFN-γ and IL-5 ELISPOT assays. Each symbol within a panel represents the combined IFN-γ/IL-5 ELISPOT data for an individual patient, with filled circles indicating patients with active disease and open inverted triangles reflecting patients that were free of disease at the time of evaluation, as described in Table I.

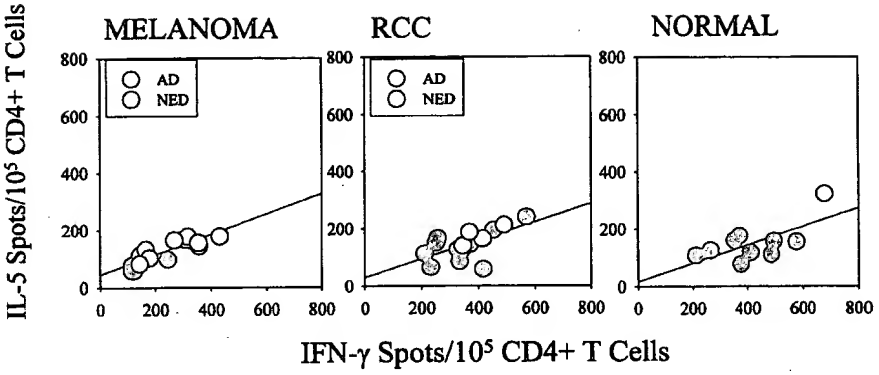

Figure 3. Patient $CD4^+$ T cells from IVS cultures exhibit normal, mixed Th1/Th2 responses to PHA mitogenic stimulation regardless of disease status. $CD4^+$ T cell cultures obtained from IVS outlined in Figs. 1 and 2 were analyzed for their response to PHA mitogen (5 μg/ml). Each symbol represents the combined IFN-γ/IL-5 ELISPOT data for an individual, with melanoma or RCC patients with active disease denoted by filled circles, and patients with no evidence of disease indicated by open circles.

stage IV disease (with the exception of patients SLM6, SLM9, and SLM23), the RCC patients were approximately equally represented by individuals with stage I or stage IV disease (with patient SLR17 exhibiting stage II disease). A comparison of whether the observed Th2-polarization in $CD4^+$ T cell response to MAGE-6 peptides (Fig. 2, top series) was correlated with disease stage in RCC patients, provided no statistically significant associations (stage I versus stage IV for MAGE-$6_{121-144}$ [$P = 0.89$]; for $MAGE_{140-170}$ [$P = 0.27$]; for MAGE-$6_{246-263}$ [$P = 0.50$]).

Patients with active disease were not predisposed to a general Th2-type polarization in their $CD4^+$ T cell responses. An evaluation of $CD4^+$ T cells from the patients with melanoma or RCC, or normal donors revealed mixed Th1-/Th2-type responses to PHA mitogenic stimulation in IFN-γ and IL-5 ELISPOT assays (Fig. 3). As groups, patients with active disease, patients with no evidence of disease and normal donors proved indistinguishable in their bulk $CD4^+$ T cell responses to mitogen. Using a series of newly defined HLA-DR4–presented helper epitopes defined from the influenza virus matrix protein ($FluM1_{60-73}$; reference 35) and EBV EBNA-1 (EBNA-$1_{519-533}$; reference 36), we were able to evaluate $CD4^+$ T cell responses in patients SLR18 and SLR19 who each presented with active disease at the time of analysis (Fig. 4). In each case, strongly Th2-type biased $CD4^+$ T cell reactivity was noted against the three MAGE-6 epitopes, with concurrent Th1-type polarized $CD4^+$ T cell antiviral responses.

Patients with RCC or Melanoma Do Not Exhibit Th3-/Tr-type $CD4^+$ T Cell Responses to MAGE-6 Epitopes. Although no ELISPOT is currently available to evaluate TGF-β production, an index for the bioactivity of the Tr-type $CD4^+$ T cells, we analyzed the supernatant from the peptide-stimulated ELISPOT wells for TGF-β levels using a cytokine specific ELISA assay. All supernatants were below the level of detection for secreted TGF-β (i.e., <60 pg/ml, unpublished data). We also evaluated these supernatants for the presence of IL-10 production and were unable to demonstrated peptide-specific secretion of this cytokine (i.e., <7 pg/ml, unpublished data). Based on direct comparison to a newly developed IL-10 ELISPOT assay, as few as 1–5 IL-10 secreting $CD4^+$ T cells (per 50,000) would have registered as 12–17 pg IL-10/ml in our ELISA assay (unpublished data). Hence we believe that these patients have, at best, very few (frequencies ≤1/50,000 $CD4^+$ T cells) in their peripheral blood.

Successful therapy associated with NED status is linked to a conversion of peripheral Th2-type to Th1-type $CD4^+$ T cell response to MAGE-6 epitopes. RCC patient SLR12 with stage I disease was surgically managed, resulting in disease-free status. Peripheral blood $CD4^+$ T cell responses were evaluated pre- and postsurgery in IFN-γ and IL-5 ELISPOT assays against the MAGE-$6_{121-144}$, MAGE-

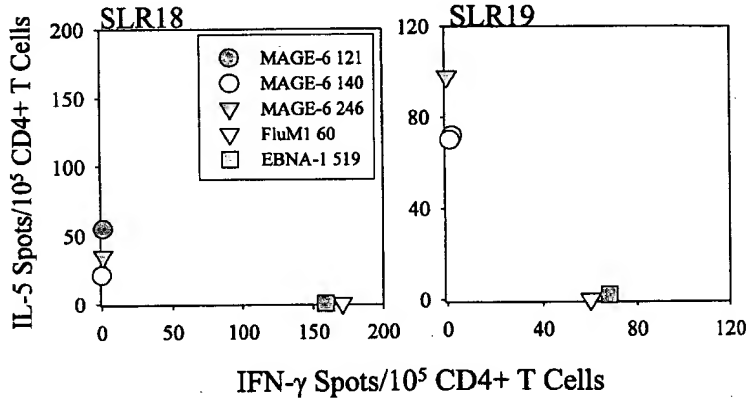

Figure 4. Peripheral blood $CD4^+$ T cells from patients SLM18 and SLM19 display Th2-type reactivity to MAGE-6 epitopes, but Th1-type reactivity to viral epitopes. $CD4^+$ T cells were stimulated with immature autologous DCs pulsed with the indicated peptides for 1 wk, as outlined in Figs. 1 and 2. Responder $CD4^+$ T cells were then analyzed for their reactivity to T2.DR4 cells pulsed with individual MAGE-6, influenza matrix, or EBV peptides in both IFN-γ and IL-5 ELISPOT assays.

$6_{140-170}$, and MAGE-$6_{246-263}$ epitopes, as outlined above. Weak Th2-biased responses were noted against the MAGE-$6_{121-144}$ and MAGE-$6_{246-263}$ peptides before surgery, while weak, but Th1-biased responses against these epitopes were noted 1 mo postsurgery (Fig. 5 A).

Patient SLM1 with stage IV melanoma was treated with an autologous DC-based vaccine (UPCI 95–060) and achieved a complete response in March 1997. Peripheral blood $CD4^+$ T cells were isolated both pre- (8/96 and 3/97) and post- (4/97, 11/98) regression of disease. Patient SLM1 was disease-free at the time of both postregression time points. As shown in Fig. 5 B, this patient reacted to all three MAGE-6 epitopes in a strongly Th2-biased manner before regression, but displayed only Th1-type reactivity after the tumor burden was clinically eradicated.

Discussion

We analyzed peripheral blood T cells harvested from HLA-DRβ1*0401$^+$ normal donors and patients with RCC or melanoma for the magnitude and nature of $CD4^+$ T cell responses to 3 MAGE-6 epitopes that we have recently identified as HLA-DRβ1*0401-presented epitopes (unpublished data). We observed a dominance of Th2-type (and a frequent lack of any Th1-type) $CD4^+$ T cell responses to these MAGE-6 epitopes in RCC and melanoma patients with active disease (Fig. 2). In marked contrast, normal donors and patients that had been successfully treated and were disease free at the time of analysis, displayed either mixed Th1-/Th2-type or strongly Th1-polarized immunity to these same peptides. It should be stressed that these polarized $CD4^+$ T cell responses are specific for the tumor peptides tested and do not reflect the general tendency of the donor to respond in a generically Th2- (or Th1-) biased fashion, as the mitogen (PHA) control spot frequencies obtained for both the IL-5 and IFN-γ ELISPOT assays were indiscriminant between patients with cancer, patients that were free of disease, and normal donors (Fig. 3). In addition, for two RCC patients (SLR18 and SLR19) evaluated, Th2-type immunity to MAGE-6 peptides coexisted with strong Th1-type immunity to influenza- and EBV-derived helper epitopes (Fig. 4). Overall, although these data derive from relatively few patients, they suggest that Th2-type dominated $CD4^+$ T cell responses against MAGE-6 epitopes may correlate with active disease status in the patient.

However, when the results of RCC patients with stage I disease were compared with those of RCC patients with stage IV disease, we were unable to determine any significant linkage between the degree of Th2-polarization to MAGE-6 epitopes and disease-stage in patients with active disease. As MAGE-6 appears to represent an early tumor-associated antigen, observed in even premalignant lesions (29–31), our results may suggest that skewing of a normally mixed Th1-/Th2-type MAGE-6-specific $CD4^+$ T cell responses toward Th2-dominated immunity may also be an early event in disease progression. Such polarization could result from chronic antigenic restimulation in situ throughout disease ontogeny, and could be initiated even in individuals with premalignant MAGE-6^+ lesions, potentially serving as a facilitator of disease progression.

Clearly, far more extensive longitudinal studies will be required to determine the prognostic significance of differential Th1- versus Th2-type in the progression, clearance, and/or recurrence of disease. Although we are now in the process of initiating these types of comprehensive studies at the UPMC and UPCI, our preliminary data provided in Fig. 5, suggests that in an RCC patient that was surgically managed and a melanoma patient that was treated with an autologous DC-based vaccine, that Th2-biased responses

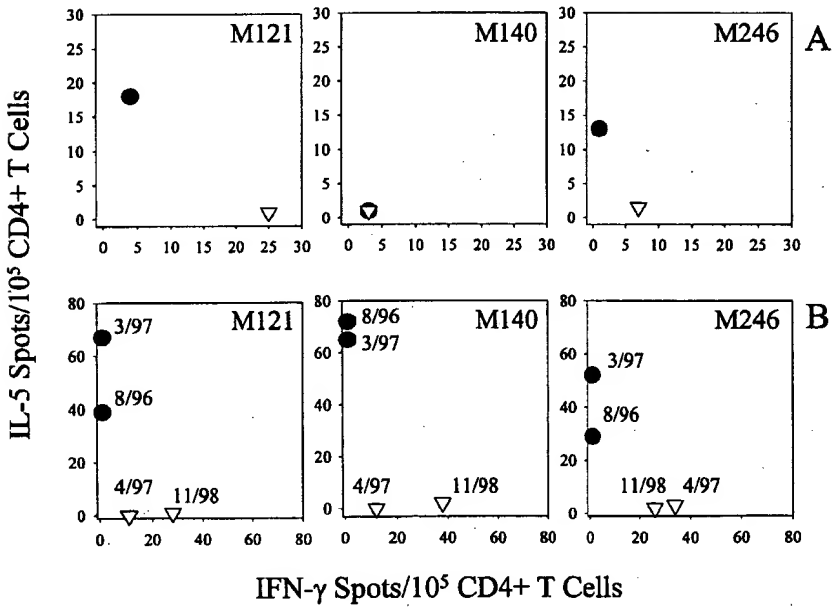

Figure 5. Analysis of the Th1-type vs. Th2-type $CD4^+$ T cell response to MAGE-6 peptides in HLA-DRβ1*0401$^+$ RCC and melanoma patients pre/posttherapy. Peripheral blood T cells were isolated from an RCC patient SLR12 (A) and melanoma patient SLM1 (B) pre- or posttherapy. In both cases, therapy resulted in disease-free status. In the case of patient SLR12, blood was drawn at the time of surgery (filled circle) and 2 mo postsurgery (open inverted triangle). Patient SLM1 was treated with a DC-based vaccine, resulting in a complete response in 3/97. Blood was drawn pretherapy and during therapy, but before regression (filled circles) and after complete regression (open inverted triangles). 7-d IVS were performed as outlined in Fig. 1, with responder $CD4^+$ T cells analyzed for their reactivity to T2.DR4 cells pulsed with individual MAGE-6 peptides in both IFN-γ and IL-5 ELISPOT assays.

to MAGE-6 epitopes shifted to Th1-biased response after the patient achieved disease-free status. Furthermore, based on preliminary MHC-peptide tetramer analysis, MAGE-6 reactive $CD4^+$ T cells were observed to increase in the peripheral blood, at least transiently, as soon as 1–2 mo after successful therapy (unpublished data), which may support the register of these helper T cell responses with clinical benefit.

An additional important consideration in our prospective analyses will be a careful comparison of systemic versus tumor-associated $CD4^+$ T cell response polarization to MAGE-6 epitopes. It would be hypothesized that the most dramatic polarizations and highest frequencies of non-Th1 polarized tumor antigen-specific $CD4^+$ T cells would be identified in the tumor microenvironment and tumor-draining lymph nodes. Although we have reported an essentially qualitative Th2-type bias in response to MAGE-6 peptides in the peripheral blood of patients with active disease, we have been thus far, unable to demonstrate systemic antigen-specific Th3/Tr-type $CD4^+$ T cell responses to these epitopes. This may suggest that these latter responses are rare-events in the patient, or alternatively, that they may be concentrated and best observed within the tumor-involved tissues of the patient.

An analysis of the data presented in Table I indicates that 12/15 (i.e., 80%) evaluable melanoma biopsies expressed the MAGE-6 antigen as deduced by RT-PCR analysis. In all 12 of these MAGE-6^+ patients, Th1-type or Th2-type $CD4^+$ T cell responses were detected by ELISPOT analysis against at least one of the three MAGE-6 epitopes analyzed in this study. In 2/3 cases where the patient's tumor failed to express the MAGE-6 gene product, the patient's $CD4^+$ T cells did not react to MAGE-6 epitopes. The resected tumor in patient SLM22, however, failed to express the MAGE-6 mRNA, yet Th2-type $CD4^+$ T cell responses were observed against all three MAGE-6–derived helper epitopes. This result may be due to MAGE-6 expression by nonresected metastatic lesions in this stage IV patient with active disease. Alternatively, the anti-MAGE-6 $CD4^+$ T cells may be reacting or cross-reacting against homologous epitopes derived from other MAGE-A family member proteins. An analysis of all MAGE-A family members suggests that this possibility would be most likely for the MAGE-$6_{121-144}$ peptide, which is identical to the homologous MAGE-3 sequence, but which differs in sequence at 3 or more key positions with all other MAGE-A members (unpublished data). This possibility is less likely for the MAGE-$6_{140-170}$ and MAGE-$6_{246-263}$ epitopes that differ from the homologous MAGE-3 sequence by two nonconservative D156S and Y249H substitutions within the putative core binding epitope (unpublished data). Additional nonconservative changes in these two epitope sequences are noted when comparing MAGE-6 to all other MAGE-A members. As corollary analyses, we will prospectively determine whether patient $CD4^+$ T cells isolated using specific HLA-DR4/MAGE-6 peptide tetramers recognize the homologous MAGE-A family sequences.

In these studies, we chose an in vitro induction assay using autologous immature monocyte-derived (i.e., myeloid) DCs that appeared not to skew the nature of the isolated $CD4^+$ T cell response to a given epitope. In preliminary studies, we analyzed freshly-isolated $CD4^+$ T cells from melanoma patients as the responders (arguably recall responses) to peptide-pulsed T2.DR4 targets in our ELISPOT assays and discerned the same cytokine (IFN versus IL-5) secretion bias, as we report after IVS. We systematically implemented the 7-d IVS (using immature myeloid DCs) protocol as this amplified the technically-detectable (ZEISS AutoImager) spot numbers in each of the assays, without changing the bias of cytokines produced. We feel that this is a reasonable amplification tool in the outlined work. As this report was designed to reflect, as closely as possible, the in situ peripheral repertoire, we have not attempted to delineate how other DC subsets or conditioning regimens alters the bias of the responder repertoire in the current manuscript. Our data, however, may suggest that immature DCs, such as have been implemented in a number of vaccine trials (43, 44), may be clinically-inferior to strong DC1-type cells (producing high quantities of IL-12; references 45 and 46) due to their comparative inability to promote strong Th1-biased immunity in the face of existing Th2-type responses.

So, why does the tumor-reactive $CD4^+$ T cell repertoire shift to a Th2-dominated phenotype in situ in cancer bearing patients? A number of nonmutually exclusive hypotheses have been proffered. These include: immune deviation via chronic antigenic stimulation and selective sensitivity of Th1-type $CD4^+$ T cells to activation-induced apoptosis (47), the promotion of DC2-type (i.e., Th2-type promoting) antigen-presenting cell function conditioned by tumor-secreted cytokines and chemokines (i.e., IL-10, TGF-β, SDF-1; references 48 and 49), and the enforced repolarization of Th1 type-responses into Th2-type responses in situ (50), among others. Using MHC/MAGE-6 peptide tetramers, we anticipate the ability to assess the proapoptotic phenotype of specific $CD4^+$ T cells in patients with active disease versus those that have achieved NED status in future studies. Our current studies investigating $CD4^+$ T cell response to viral epitopes suggest that patient DCs do not exert a dominant DC2 functional phenotype in vitro. Clearly these important issues demand intense prospective evaluation.

Although we are currently analyzing DC-based vaccines that are capable of repolarizing Th2-type tumor-reactive $CD4^+$ T cells toward Th1-type immunity, this may not necessarily represent the clinically preferred modality for the treatment of existing disease. As previously mentioned, Th2-type tumor-specific $CD4^+$ T cells may well prove productive collaborators to Th1-type $CD4^+$ T cells in mediating tumor regression via different, but complementary mechanisms (9–12, 14, 20). This may be particularly important in the case of MHC class I–loss variant tumors that will be impervious to Th1-type $CD4^+$ T cell-sponsored cytotoxicity mediated by $CD8^+$ antitumor T cells (10, 11). Regardless of which underlying mechanism leads to tumor regression, it appears clear that strong Th1-type tumor-spe-

cific T cell responses will be important in the maintenance of durable cellular immunity (12) and extended disease-free intervals in those patients at high-risk for recurrence.

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Identification of a Promiscuous T-Cell Epitope Encoded by Multiple Members of the *MAGE* Family[1]

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ABSTRACT

One of the major limitations of tumor-specific vaccination is the generation of antigen-loss variants that are able to escape the immune response elicited by a monoantigenic peptide epitope. Here, we report the identification of a new HLA-B*3701-restricted epitope shared by four different members of the *MAGE* family. Peripheral blood lymphocytes isolated from a melanoma patient were stimulated *in vitro* with the autologous HLA-negative melanoma line transfected with autologous HLA B*3701 molecule. This protocol led to the induction of tumor-specific, B*3701-restricted CTLs specific for a peptide epitope encoded by codons 127–136 of the gene *MAGE-1*. The same epitope is also encoded by the homologous region of three other members of the *MAGE* family, *MAGE-2*, *-3*, and *-6*. Consistent with the notion that the peptide encoded by *MAGE-1* codons 127–136 is, indeed, processed from the proteins encoded by all four *MAGE* family members, the CTLs were able to specifically recognize Cos-7 cells cotransfected with HLA-B*3701 and any of these *MAGE* genes. Moreover, the CTLs also recognized a *MAGE-6*-positive melanoma line transfected with the B*3701 molecule. These findings allow the inclusion of a new set of tumor patients into clinical cancer vaccination trials. Furthermore, they suggest that some promiscuous peptide epitopes shared by different members of the *MAGE* family might be less prone to escape the immune response by generation of *MAGE* antigen loss variants.

INTRODUCTION

A number of studies have demonstrated that CTLs recognizing human tumor cells can be isolated from PBLs[3] and tumor-infiltrating lymphocytes of cancer patients (1). An *in vivo* role of such tumor-specific effectors is suggested by the results of several immunotherapy trials, mainly performed in melanoma patients (2–5).

Over the last few years, the use of such tumor-specific effectors has allowed the isolation of several genes encoding tumor antigens (6). According to the pattern of expression in neoplastic and normal tissues, these antigens can be classified into four classes, which have different degrees of tumor specificity and clinical relevance. The first class comprises antigens encoded by genes expressed in various tumors of different histotypes but not in normal tissues, other than testis and placenta, such as *MAGE*, *GAGE*, and *BAGE* (7–9). The second class represents differentiation antigens that are only expressed in melanoma and melanocytes, such as *tyrosinase*, *Melan-A/MART-1*, *gp100*, *TRP-1*, and *TRP-2* (10–15). The antigens belonging to the third class are generated by point mutations in genes that are ubiquitously expressed (16–18). The fourth class of antigens, which has been defined only recently, is represented by *TRP-2-INT2*, an antigen shared between melanomas but not expressed in normal cells of the melanocytic lineage (19).

Tumor antigens belonging to the *MAGE* family have especially elicited considerable interest because six of them, *MAGE-1*, *-2*, *-3*, *-4*, *-6*, and *-12*, are expressed selectively by a significant proportion of primary and metastatic tumors, including melanomas, lung, bladder, ovarian, and breast carcinomas (7, 20–23).

Clinical trials of vaccination, based on the use of antigenic peptides encoded by *MAGE-1* and *-3*, are in progress in patients affected by melanoma and other neoplastic diseases (3). Nevertheless, the use of *MAGE-2*, *-4*, and *-6* proteins as targets for tumor-specific immunotherapy has been hampered by the uncertainty as to whether these proteins can be recognized by specific CTLs of the immune system.

Potential limitations to the wide application of this therapeutic approach are the limited number of characterized CTL epitopes (*i.e.*, tumor antigen peptides and appropriate HLA class I alleles) and the *in vivo* generation of antigen loss variants that are able to escape the immune response elicited by a monoantigenic vaccine (24). Indeed, such clinical protocols apply only to patients carrying a tumor expressing a well-known tumor antigen and a defined HLA allele. Unfortunately, a large majority of cancer patients do not fulfill these including criteria. Therefore, identification of new antigenic determinants is a priority because it would increase the number of patients that could benefit from antitumor vaccination protocols.

Here, we report the identification of a new HLA-B*3701-restricted epitope, encoded by homologous regions of the *MAGE-1*, *-2*, *-3*, and *-6* genes. The use of identical antigenic peptides derived from different highly homologous proteins, in protocols of vaccination, may represent a useful tool to avoid the generation of antigen loss variants. Moreover, this study presents, for the first time, evidence for the existence of human CTL recognizing peptides derived from MAGE-2 and -6 proteins, which now can be included in the list of possible antigens for targeted immunotherapy of neoplastic disorders.

MATERIALS AND METHODS

Cell Lines. The melanoma cell line MSR3-mel was established in our laboratory from a metastatic lesion of patient MSR3 and cultured in IMDM supplemented with 10% FCS. PBLs of this patient were serologically typed as: HLA-A1, A11, B37, B5, Cw6. The melanoma line MZ2-MEL.2.2 ET.1 (HLA-A1, B*3701, Cw6), hereafter referred to as ET1, and the Cos-7 cell line were kindly provided by Prof. T. Boon (Ludwig Institute for Cancer Research, Brussels, Belgium) and maintained in DMEM supplemented with 10% FCS. Me14932 (HLA-A2/A3, B7/Bw50, Cw6/Cw7) was a kind gift of Dr. G. Parmiani (Istituto dei Tumori, Milano, Italy). The B-lymphoblastoid cell line LG2-EBV was kindly provided by Prof. T. Boon, whereas the MSR3-EBV was derived by transformation of peripheral blood B lymphocytes from patient MSR3 with the B95-8 strain of EBV.

Synthetic Peptides. Synthetic peptides were purchased from Primm (Milano, Italy). Peptides were: $MAGE_{127-136}$ (REPVTKAEML), encoded by codons 127–136 of *MAGE-1*, *-2*, *-3*, and *-6* genes; and $M4_{127-136}$ (KELVTKAEML) and $M12_{127-136}$ (REPFTKAEML), corresponding to amino acids 127–136 encoded by genes *MAGE-4* and *-12*, respectively. Peptides were dissolved to 10 mM in DMSO and diluted further in 0.9% NaCl.

Subcloning of the *HLA-B*3701* Allele. Total RNA was prepared from MSR3 PBLs by the RNeasy Total RNA Kit (Qiagen, Hilden, Germany). cDNA corresponding to 300 ng of total RNA was amplified by PCR using a primer pair suitable for specific amplification and directional cloning of the full-length

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[3] The abbreviations used are: PBL, peripheral blood lymphocyte; IMDM, Iscove's modified Dulbecco's medium; mAb, monoclonal antibody; IL, interleukin; LNGFR, low-affinity nerve growth factor receptor; β2-m, β2-microglobulin.

coding region of *HLA-B* alleles (generous gift of Dr. Soo Young Yang, Memorial Sloan-Kettering Cancer Center, New York, NY). The 1.1-kb PCR product was subcloned into the eukaryotic expression vector pcDNA3.1 (Invitrogen Corp., Oxon, United Kingdom). Plasmid clones encoding HLA-B*3701 and -B*52011 (the *HLA-B37* and *-B5* alleles of patient MSR3) were identified using diagnostic restriction enzymes. The *HLA-B*3701* gene was then sequenced to verify the correspondence to the published DNA sequence. This plasmid was called pcDNA3.1/B*3701.

Transfection of Melanoma Cell Lines. Melanoma cell lines were transfected by the calcium phosphate precipitation technique with pcDNA3.1/HLA-B*3701 and selected in G418. Expression of the transfected HLA-B*3701 molecule in stable transfectants was verified by flow cytometry with the HLA-A-, HLA-B-, and HLA-C-specific mAb W6/32.

***In Vitro* Induction of CTL Line 337.** CTL line 337 was obtained using a protocol previously described by others (25), with slight modifications. Briefly, PBLs from patient MSR3 were separated by Ficoll gradient and cultivated (1×10^6–2×10^6 cells/well) with the autologous, irradiated MSR3-B37 melanoma cells (0.5×10^5–1×10^5 cells/well) in 2 ml of IMDM supplemented with 10% human serum, glutamine, and antibiotics. After 3 days of culture, 10 units/ml IL-2 (Chiron, Milan, Italy) and 5 ng/ml IL-7 (Genzyme Corp., Cambridge, MA) were added. Lymphocytes were restimulated weekly with 0.5×10^5 irradiated MSR3-B37 cells and tested in a cytotoxicity assay after three stimulations. After the fifth restimulation, 2×10^6 irradiated LG2-EBV cells were added as feeder cells, and IL-2 was increased to 50 units/ml.

Assay for Cytolytic Activity and Peptide Binding Studies. Lytic activity of the cytotoxic T-cell lines was tested in a chromium release assay as described previously (26). Peptides were tested in chromium release assays: ^{51}Cr-labeled target cells were incubated for 1 h at room temperature in 96-well microplates with various concentration of the peptide before addition of effector cells at a fixed E:T ratio. Binding of peptides $M4_{127-136}$ and $M12_{127-136}$ to the HLA-B*3701 molecule was studied in a competition assay, as described previously (27). As standard peptide, we used peptide $MAGE_{127-136}$ (300 nM), recognized by CTL 337. CTLs were used at an E:T ratio of 30:1.

Production of Subfragments of *MAGE-1*. Subfragments of *MAGE-1* gene (495- and 1072-bp fragments) were obtained by digestion of *MAGE-1* cDNA with *Bgl*II and *Eco*RI. After purification on agarose gel, the fragments were cloned into the pcDNA3.1 plasmid. Clones were isolated, plasmid DNA was extracted and transfected into Cos-7 cells along with the *HLA-B*3701* gene.

Transfection of Cos-7 Cells and IFN-γ Release Assay. Transfection of Cos-7 cells was performed by the DEAE-dextran-chloroquine method (12). Briefly, 1.5×10^4 Cos-7 cells were transfected with 100 ng of plasmid pcDNA3.1/B*3701 and 100 ng of expression vectors containing the cDNA of one of the following genes: *MAGE-1*, *-2*, *-3*, *-4*, *-6*, and *-12*. Transfected Cos-7 cells were tested in a IFN-γ assay after 48 h: 5000 responder CTLs, at day 5 after stimulation, were added in 150 μl of IMDM-10% human serum supplemented with 25 units/ml IL-2. After 24 h at 37°C, 100 μl of supernatant were harvested, and the IFN-γ concentration was measured using a IFN-γ release kit (Genzyme Corp.) according to the manufacturer's recommendations.

Retroviral Vector-mediated Gene Transfer of *HLA-B*3701* into Me14932. The retroviral vector B37-CSM, coding for the HLA-B*3701 molecule of patient MSR3, was constructed as described previously (28). Briefly, the full-length cDNAs coding for the HLA-B*3701 molecule was cloned under the control of the viral long terminal repeat, whereas the truncated form of the human LNGFR (ΔLNGFR) was driven by the SV40 promoter. The ecotropic murine fibroblast cell line GP+E86 was transiently transfected with 30 μg of retroviral construct by standard calcium-phosphate method. Infection of the amphotropic murine packaging cell line GP+env Am 12, by supernatant of 48 h cultures of transfected GP+E86 cells, was performed for 4 h in the presence of 8 mg/ml polybrene. Infected packaging cells were immunoselected for ΔLNGFR expression by magnetic beads (Dynabeads M-450; Dynal A.S., Oslo, Norway) coated with the LNGFR-specific mAb 20.4 (American Type Culture Collection, Rockville, MD). Transduction of Me14932 was performed by cultivation with retrovirus-containing supernatant in the presence of polybrene (8 mg/ml). Five or six rounds of infection of at least 4 h were performed. Efficiency of infection was evaluated by immunofluorescence analysis with the LNGFR-specific mAb 20.4 and with a HLA-Bw4-specific mAb.

RT-PCR Assays. *MAGE-1*, *-2*, *-3*, *-4*, *-6*, and *-12* and *β2-m* cDNAs were detected by PCR amplification. Reaction mixture contained 5 μl of cDNA suspension, 4 μl of a 10 mM dNTPs mixture (containing each dNTP at 2.5 mM), 5 μl of 10× DNA polymerase buffer (Finnzymes Oy, Espoo, Finland), 2 units of DynaZyme DNA polymerase (Finnzymes Oy), and sterile distilled water up to a 50-μl total reaction volume. For oligonucleotide primer sequences and PCR amplification programs, see Weynants *et al.* (Ref. 20; MAGE-1, -2, and -3) and De Plaen *et al.* (Ref. 29; MAGE-4, -6, and -12). β-2m cDNA was amplified using the sense primer β 5′ (5′-AAC CAC GTG ACT TTG TCA CAG C-3′) and antisense primer β 5′ (5′-CTG CTC AGA TAC ATC AAA CAT G-3′). PCR amplification was performed for 30 cycles (1 min at 94°C, 30 s at 56°C, and 2 min at 72°C); the expected length of β-2m amplification product was 230 bp. RNA integrity was tested by PCR with β-actin-specific oligonucleotide primers (30). Samples scored positive when a band of the appropriate size was visible on a agarose gel in the presence of ethidium bromide.

RESULTS

MSR3-B37 Induces an Antigen-specific Immune Response. The melanoma line MSR3 was established from a cutaneous metastasis resected from patient MSR3. Expression of HLA class I alleles by the tumor cells was barely detectable (Fig. 1) and appeared to be inadequate to allow antigen presentation to immune effectors. Indeed, the MSR3 melanoma line failed to induce a cytotoxic response from autologous PBLs (data not shown). The lack of class I cell surface expression by MSR3-mel was not caused by impaired β-2m synthesis because a β-2m-specific mRNA was detected by RT-PCR analysis (data not shown).

To determine whether HLA class I antigen expression could be restored, MSR3-mel cells were stably transfected with cDNA encoding the autologous HLA-B*3701 molecule. After G418 selection flow cytometric analysis showed staining of the transfected MSR3-B37 cell line by the W6/32 mAb (Fig. 1).

To evaluate the presence on the surface of MSR3-B37 line of tumor-specific antigens, the melanoma cells were tested for their ability to induce tumor-specific cytotoxic effectors and for their susceptibility to lysis by these CTLs. Patient's PBLs were *in vitro* stimulated by MSR3-B37 as described in "Materials and Methods." After three rounds of stimulation, the polyclonal cytotoxic T cell line 337 (CTL 337) specifically lysed the MSR3-B37 cell line but not the untransfected MSR3-mel (Fig. 2). Autologous MSR3-EBV cells and PHA-activated T blasts were not recognized (data not shown), suggesting that the epitopes recognized by these CTLs are melanoma-/melanocyte-specific. Indeed, in addition to the autologous melanoma cells, CTL 337 also lysed the HLA-B*3701-positive melanoma line ET1 (Fig. 2), suggesting that one or more shared melanoma antigens are recognized.

These data indicate that HLA class I expression can be restored by transfection of MSR3 melanoma cells and that the melanoma line transfected with the HLA-B*3701 molecule is able to induce a tumor-specific cytotoxic T-cell response.

Identification of the Antigenic Epitope Recognized by CTL 337. To identify the antigen recognized by CTL 337, we evaluated the IFN-γ release of CTL 337 in the presence of Cos-7 cells transfected with plasmid pcDNA3.1/B*3701, along with cDNA encoding six members of the *MAGE* family (*i.e.*, *MAGE-1*, *-2*, *-3*, *-4*, *-6*, and *-12*), some of which are expressed by both MSR3-mel and ET1. CTL 337 specifically recognized Cos-7 cells transfected with *MAGE-1*, *-2*, *-3*, and *-6* (Fig. 3), suggesting that the epitope target of CTL 337 was shared among the four different antigens or that distinct components of the oligoclonal T-cell line were recognizing peptides derived from the four *MAGE* gene products. A low level of IFN-γ was detected in

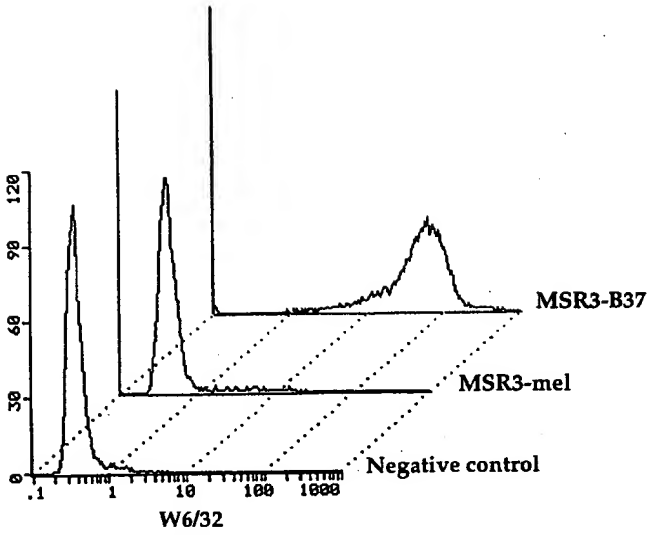

Fig. 1. Expression of HLA-class I molecules by MSR3-mel and MSR3-B37. Tumor cells were incubated with mAb W6/32 (anti-HLA-class I) or with an isotype control, washed, and labeled with goat antimouse immunoglobulin antibodies coupled to fluorescein. The analysis was performed before and after HLA-B*3701 transfection of MSR3-mel.

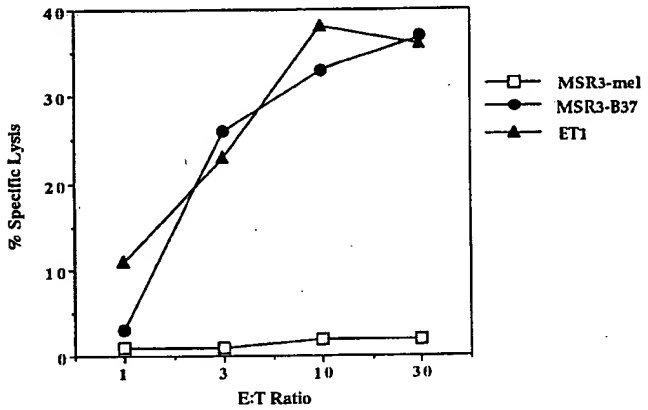

Fig. 2. Recognition of a shared HLA-B*3701-restricted antigen by CTL 337. The cytotoxic activity of CTL 337 cells was assessed against the autologous melanomas MSR3-mel and MSR3-B37 and against the allogenic melanoma ET1 at various E:T ratios.

the presence of *MAGE-4*- and *MAGE-12*-transfected Cos-7 cells (Fig. 3).

To identify the sequence coding for the antigenic peptide(s) recognized by CTL 337, we digested cDNA encoding *MAGE-1* with *Bgl*II and *Eco*RI obtaining two subfragments of ~495 and 1072 bp (Fig. 4). They were cloned into plasmid pcDNA 3.1 and transfected into Cos-7 cells along with HLA-B*3701 molecule. The presence of an in-frame start codon at 202 and 707 bp in the 495- and 1072-bp fragments, respectively, assured the expression of the two subfragments in the transfected cells. The level of IFN-γ released by CTL 337 cells in the presence of Cos-7 cells transfected with the 495-bp fragment was comparable to that conferred by the entire *MAGE-1* gene (Fig. 4), indicating that the antigenic peptide was encoded within this region. The amino acid sequence encoded by the 495-bp fragment (Fig. 5) was screened for peptides carrying the binding motif for HLA-B*3701 (31). Five peptides carrying aspartate or glutamate in position 2 and isoleucine or leucine in position 9/10 were identified (Fig. 5). One of these peptides, REPVTKAEML, was present also in the amino acid sequences encoded by *MAGE-2*, *MAGE-3*, and *MAGE-6*. This peptide, denominated MAGE.127–136, was used to sensitize the MSR3-EBV line to lysis by CTL 337 cells in a titration assay (Fig. 6*A*). The half-maximal lysis was reached with 90 nM peptide. No lysis of MSR3-EBV pulsed with an unrelated peptide that was able to bind to HLA-B*3701 was observed (Fig. 6*B* and data not shown).

Low levels of IFN-γ were released by the CTL 337 cells in the presence of Cos-7 cells expressing *MAGE-4* and *-12* (Fig. 3). To verify whether this release could be ascribed to recognition of peptides encoded by codons 127–136 within *MAGE-4* and *MAGE-12*, a peptide-binding study was performed, using MSR3-EBV cells pulsed with the two peptides as targets. Peptide $M4_{127-136}$, KELVTKAEML, differs by two amino acids (lysine *versus* arginine in position 1 and leucine *versus* proline in position 3) from peptide REPVTKAEML, whereas peptide $M12_{127-136}$, REPFTKAEML, differs by only one amino acid (phenyalanine *versus* valine in position 4). The results revealed that the two peptides can bind to HLA-B*3701 because increasing amounts of both were able to inhibit the lysis of MSR3-EBV pulsed with peptide REPVTKAEML but not with an unrelated HLA-A1-binding peptide (*i.e.*, M3.271–279; Fig. 6*B*). However, no recognition of EBV cells pulsed with peptides $M4_{127-136}$ and $M12_{127-136}$ was observed (data not shown).

Taken together, these data indicate that CTL 337 cells are able to recognize a peptide endogenously processed from *MAGE-1*, *-2*, *-3*, and *-6* products. The two peptides, encoded by the same region of *MAGE-4* and *-12*, respectively, are able to bind to HLA-B*3701, but they are not recognized by CTL 337 cells.

CTL 337 Cells Specifically Recognize *MAGE-2* and *-6* Gene Products. Until now, there was no evidence of the immunogenicity of *MAGE-2*- and *MAGE-6*-encoded proteins in humans. Indeed, peptides encoded by *MAGE-1*, *-3*, *-4*, and *-12* have been found to bind to various class I molecules to form antigens recognized by different CTLs, whereas no peptides encoded by the genes *MAGE-2* or *MAGE-6* have thus far been identified.

To demonstrate that peptide REPVTKAEML could also be processed from *MAGE-2* and *-6* and presented to CTL 337 cells, we attempted to look for melanoma cell lines expressing *MAGE-2* or *-6* but none of the other *MAGE* genes. Unfortunately, expression of the *MAGE* genes in melanomas is strictly correlated: most of the melanomas that express one member of the *MAGE* gene family also express the others. Indeed, we were unable to find a melanoma line

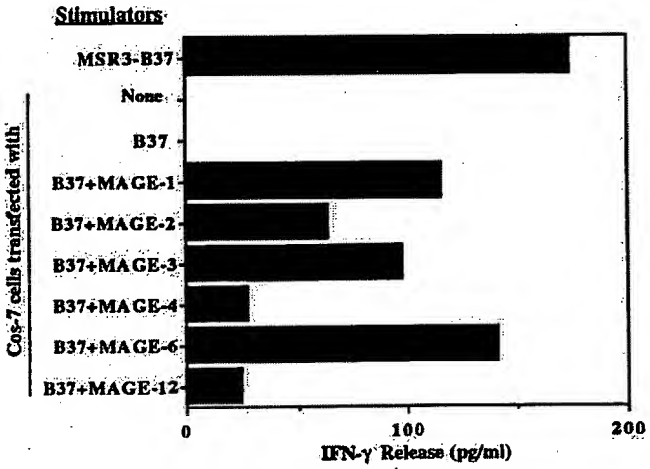

Fig. 3. Identification of the tumor-antigens recognized by CTL 337. Cos-7 cells were cotransfected with HLA-B*3701, alone or together with cDNAs encoding genes *MAGE-1*, *-2*, *-3*, *-6*, and *-12*. After 48 h, CTL 337 cells were added, and the IFN-γ released was measured 24 h later, as described in "Materials and Methods." MSR3-B37 was included as positive control.

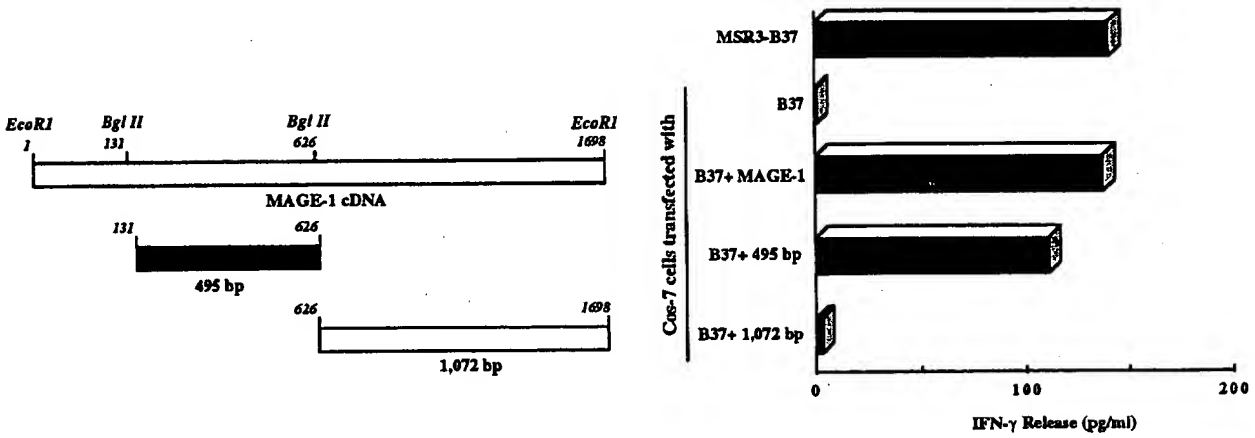

Fig. 4. Identification of the sequence coding for the antigenic peptide recognized by CTL 337. Cos-7 cells were cotransfected with two subfragments of *MAGE-1* cDNA together with HLA-B*3701. After 48 h, CTL 337 cells were added, and production of IFN-γ was measured 24 h later, as described in "Materials and Methods." As control, Cos-7 cells were transfected with HLA-B*3701, alone or together with the full-length *MAGE-1* cDNA.

that selectively expresses *MAGE-2*, but we succeeded in finding a single melanoma line, Me14932, that selectively expresses *MAGE-6* at low level (data not shown).

To verify whether peptide REPVTKAEML is endogenously processed from *MAGE-6* products and presented by HLA-B*3701, Me14932 was transduced by a retroviral vector encoding the HLA-B*3701 molecule. As indicated by immunofluorescence staining with a HLA-Bw4-specific mAb, cell surface expression of HLA-B*3701 on a pure population of transduced Me14932 cells was at least 2-fold lower than that of MSR3-B37 melanoma cells (data not shown). CTL 337 cells were able to recognize the Me14932-LB37 line in a cytotoxicity assay, and the level of lysis was increased by the exogenous addition of peptide REPVTKAEML, whereas there was no recognition of the pulsed and unpulsed Me14932 lines (Fig. 7). The low levels of lysis of the melanoma Me14932-LB37 might be explained either by weak expression of gene *MAGE-6* and by the weak surface expression of HLA-B*3701 molecules.

To evaluate whether the inclusion of *MAGE-2* and *-6* in the list of possible target antigens for specific immunotherapy could increase the proportion of eligible patients, we analyzed the expression of *MAGE-1*, *-2*, *-3*, and *-6* in fresh tumor samples of various histotypes. Melanomas were not analyzed because expression of the different *MAGE* genes was clearly correlated (32). The results indicate that 12% of the ovarian carcinomas and 5% of colon and breast carcinomas express *MAGE-2* and/or *-6* in the absence of *MAGE-1* and *-3* (Table 1). On the other hand, in all bladder and lung carcinomas studied the four genes were always coexpressed.

In conclusion, the data reported in this study indicate that *MAGE-2* and *-6* can be included in the list of possible target antigens for

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gaagatctgc ctgtgggtct tcattgccca gctcctgccc acactcctgc ctgctgccct gacgagagtc atcatgtctc ttgagcagag 221

                                L   E A Q   Q E A L   G L
gagtctgcac tgcaagcctg aggaagccct tgaggcccaa caagaggccc tgggcctggt gtgtgtgcag gctgccacct cctcctcctc 311

tcctctggtc ctgggcaccc tggaggaggt gcccactgct gggtcaacag atcctcccca gagtcctcag ggagcctccg cctttcccac 401

                                                        E E   G P S   T S C I / L E S
taccatcaac ttcactcgac agaggcaacc cagtgagggt tccagcagcc gtgaagagga ggggccaagc acctcttgta tcctggagtc 491

 L F R   A V I                  A D L   V G F L   L L                  R E P V   T K A
cttgttccga gcagtaatca ctaagaaggt ggctgatttg gttggttttc tgctcctcaa atatcgagcc agggagccag tcacaaaggc 581

 E M L
agaaatgctg gagagtgtca tcaaaaatta caagcactgt tttcc                                              671
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Mage-1	REPVTKAEML
Mage-2	REPVTKAEML
Mage-3	REPVTKAEML
Mage-4	KELVTKAEML
Mage-6	REPVTKAEML
Mage-12	REPFTKAEML

Fig. 5. *Top*, sequence of the 495-bp subfragment of *MAGE-1* cDNA. Peptides carrying the binding motifs for HLA-B*3701 are listed above their respective nucleotide sequences. *Bottom*, comparison of peptide REPVTKAEML (*underlined*), encoded by *MAGE-1*, with the peptides encoded by the homologous regions of other genes of the *MAGE* family.

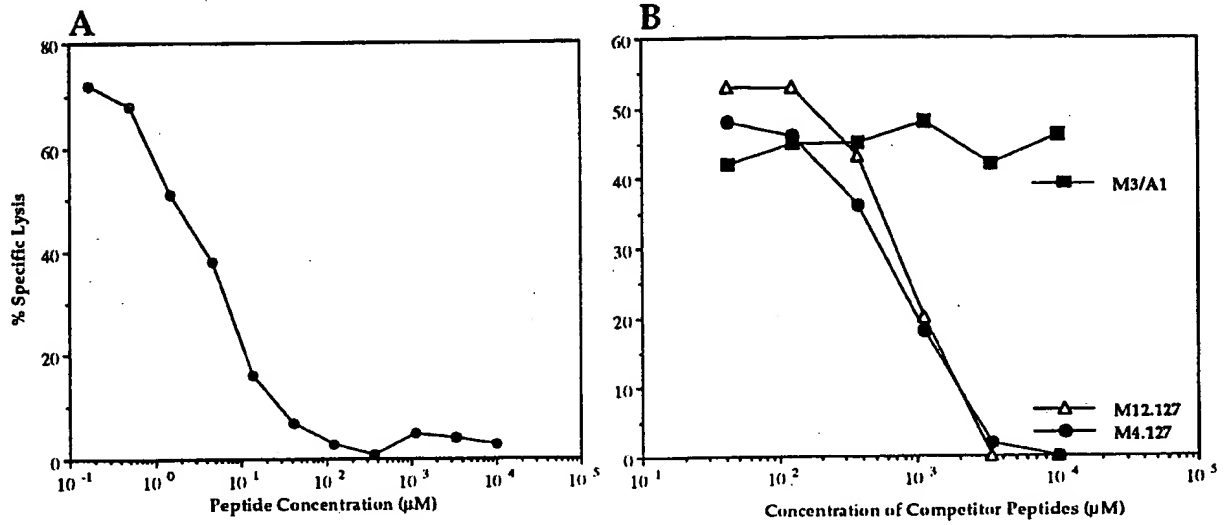

Fig. 6. *A*, recognition of the peptide $MAGE_{127-136}$ by CTL 337 cells. MSR3-EBV cells were incubated with 3-fold dilutions of peptide $MAGE_{127-136}$, starting from 10 mM and used as target cells in a standard cytotoxicity assay. The E:T ratio was fixed at 10:1. *B*, binding of peptides $M4_{127-136}$ and $M12_{127-136}$ to HLA-B*3701, evaluated in a competition assay. Competitor peptides included the $M4_{127-136}$ peptide KELVTKAEML and the $M12_{127-136}$ peptide REPFTKAEML. The M3.A1 (*i.e.*, $M3_{271-279}$) peptide, which was unable to bind to the HLA-B*3701 molecule, was used as negative control. Percentage lysis without competitor peptides was 52%.

tumor-specific immunotherapy, increasing the number of patients that could benefit from this therapy.

DISCUSSION

In the last few years, there has been a considerable effort to characterize antigenic peptides encoded by tumor-associated antigens and the HLA molecules responsible for their presentation (6). Several immunotherapy clinical trials of cancer vaccinations based on the use of these peptides are in progress, with quite positive preliminary results. Indeed, some objective cancer responses have been observed, consisting of both tumor regression (3, 5) and a few long-term complete responses (4).

One major limitation outlined by those studies is the development, in a significant proportion of the treated patients, of tumor variants that fail to express the antigen recognized by tumor-reactive lymphocytes (24). Those variants can be generated either by loss of the nominal antigen (referred to as antigen loss variants; Refs. 33 and 34) or by molecular defects affecting different steps

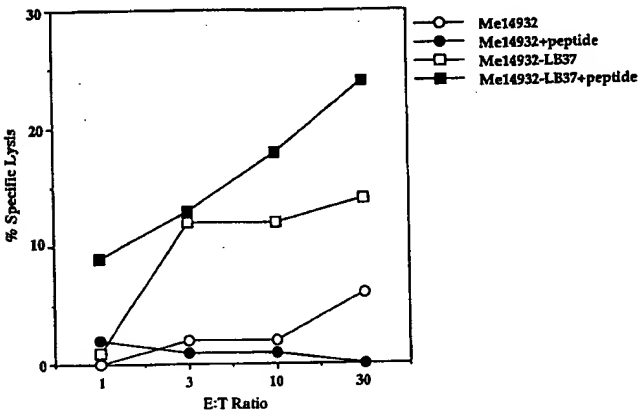

Fig. 7. Recognition of a *MAGE-6*-positive melanoma cell line by CTL 337. The HLA-B*3701-negative line Me14932 and the HLA-B*3701-positive line Me14932-LB37 were pulsed or not with 16 μM peptide $MAGE_{127-136}$ and used as target cells in a standard cytotoxicity assay at the indicated E:T ratios.

Table 1 *Expression of MAGE genes by fresh tumor samples*[a]

	% RT-PCR-positive tumors				
Histological type	*MAGE-1*	*MAGE-3*	*MAGE-2*	*MAGE-6*	*MAGE-2* or *MAGE-6* only
Lung carcinoma (28)[b]	35	39	32	29	0
Breast carcinoma (20)	30	10	10	15	5
Ovary carcinoma (25)	24	20	32	20	12
Bladder carcinoma (25)	28	28	20	24	0
Colon carcinoma (17)	0	5	5	5	5

[a] As determined by reverse transcriptase-PCR (RT-PCR) analysis.
[b] Numbers in parentheses represent numbers of fresh tumor samples analyzed.

of the antigen presentation pathway (referred to as presentation loss variants; Refs. 35–37). An active intervention of the immune system in the selection of antigen loss and presentation loss variants have been observed in both treated (24) and untreated patients (33, 38). However, escape from classical tumor-specific CTLs may be counteracted *in vivo* by the intervention of different immune effectors. Indeed, tumor cells that have lost expression of some but not all HLA class I molecules can be recognized by a new category of antitumor lymphocytes expressing killer-cell inhibitory receptors (39), whereas HLA-negative tumor cells can be targeted by NK cells.

The melanoma cell line used in this study belongs to the presentation loss variant class of HLA-negative tumor cells. The molecular defect responsible for the HLA class I phenotypes of MSR3-mel has not yet been identified; however, our melanoma line exhibits barely detectable levels of HLA class I expression by immunofluorescence analysis, which are not sufficient for stimulation of a tumor-specific T-cell response (data not shown). This altered phenotype does not seem to be due to β-2m or TAP alterations or to deletions of MHC genes but rather to a defect in the transcriptional machinery. Indeed, HLA class I expression in MSR3-mel can be restored by transfection of cDNAs encoding autologous HLA class I alleles.

The HLA-B*3701-transfected cell line (*i.e.*, MSR3-B37) allowed the isolation of HLA-B*3701-restricted and tumor-specific CTLs that recognized a nonapeptide encoded by the same region (*i.e.*, residues 127–136) of MAGE-1, -2, -3, and -6 proteins.

To our knowledge, this is the first B*3701-restricted tumor-specific epitope that has been identified thus far. Note that the HLA-B*3701 molecule is present on both lymphocytes and tumor cells of patient MZ2 (7), from which a large variety of *MAGE*-specific CTL clones restricted by different HLA class I molecules were isolated (8–9, 40–42). Those results suggest a subdominant role of HLA-B*3701 in tumor antigen presentation in the MZ2 model that should be overcome in the MSR3 system by the absence on the stimulating cells of a HLA class I molecule other than HLA-B*3701. Indeed, dominance of a given HLA molecule in the tumor-specific stimulation of autologous CTL by melanoma cells has been described in several model systems (39, 43, 44).

Several members of the MAGE gene family are specifically expressed by tumors of various histological types and T-cell defined epitopes encoded by *MAGE-1* and *-3* have been identified. However, although *MAGE-2* and *-6* are expressed in a large percentage of tumor samples, thus far no *MAGE-2*- and *MAGE-6*-specific CTLs have been isolated. The only suggestion that *MAGE-2* behaves like a tumor-antigen comes from the study of Visseren *et al.* (45), who demonstrated the immunogenicity of *MAGE-2* in a HLA-A*0201Kb transgenic mouse model. Therefore, our study reports the first evidence for an immunogenic potential of *MAGE-2* and *-6* in humans. Indeed, CTL 337 cells were able to recognize Cos-7 cells transfected with HLA-B*3701 and *MAGE-2* or *-6* genes. Moreover, a stable HLA-B*3701-positive melanoma line expressing *MAGE-6* was recognized, whereas analogous experiments on *MAGE-2* and HLA-B*3701-positive melanoma lines could not be performed. It has been suggested that the proteasome specifically digests proteins into polypeptides with defined hydrophobic, basic, or acidic COOH termini, whereas the NH_2-terminal cleavage into smaller fragments occurs nonspecifically 8–10 amino acids further upstream. In view of the presence of hydrophobic residues (M and L) at the COOH-terminus of peptide REPVTKAEML, as well as the high degree of the amino acid sequence homology between *MAGE-2* and *MAGE-6* in the region around peptide REPVTKAEML, it is tempting to speculate that this peptide might indeed be processed in melanoma cells also from *MAGE-2* products. (46, 47).

The molecular analysis performed on tumor samples of various histotypes revealed a strong correlation between the expression of different *MAGE* genes. However, inclusion of *MAGE-2* and *MAGE-6* in the list of target antigens for cancer immunotherapy has practical implications for the enrollment of patients with ovarian carcinomas. Indeed, 12% of the ovarian carcinoma samples analyzed express *MAGE-2* and/or *-6*, without expressing *MAGE-1* and/or *-3* genes. On the other side, coexpression of more than one *MAGE* gene by a given tumor might prevent the development of antigen loss variants during vaccination treatment. Indeed, immune escape from a peptide-induced antitumor response might then be rare, because it would require the occurrence of several independent molecular alterations.

In conclusion, the identification of this new HLA-B*3701-restricted epitope not only increases the number of patients eligible for immunization but also may prove highly efficient for immunotherapy because of reduced risk of tumor escape due to the emergence of antigen loss variants.

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ORIGINAL PAPER

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Structure, chromosomal localization, and expression of 12 genes of the *MAGE* family

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Abstract We reported previously that human gene *MAGE-1* directs the expression of a tumor antigen recognized on a melanoma by autologous cytolytic T lymphocytes. Probing cosmid libraries with a *MAGE-1* sequence, we identified 11 closely related genes. The analysis of hamster-human somatic cell hybrids indicated that the 12 *MAGE* genes are located in the q terminal region of chromosome X. Like *MAGE-1*, the 11 additional *MAGE* genes have their entire coding sequence located in the last exon, which shows 64%–85% identity with that of *MAGE-1*. The coding sequences of the *MAGE* genes predict the same main structural features for all MAGE proteins. In contrast, the promoters and first exons of the 12 *MAGE* genes show considerable variability, suggesting that the existence of this gene family enables the same function to be expressed under different transcriptional controls. The expression of each *MAGE* gene was evaluated by reverse transcription and polymerase chain reaction amplification. Six genes of the *MAGE* family including *MAGE-1* were found to be expressed at a high level in a number of tumors of various histological types. None was expressed in a large panel of healthy tissues, with the exception of testis and placenta.

The nucleotide sequence data reported in this paper have been submitted to the GenBank nucleotide sequence database and have been assigned the accession numbers U10685-U10694

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Introduction

By cultivating blood lymphocytes of cancer patients in the presence of their tumor cells, it is often possible to obtain cytolytic T lymphocytes (CTL) that show specificity for the tumor cells (Mukherji and MacAlister 1983; Knuth et al. 1984; Anichini et al. 1987). Using lymphocytes from melanoma patient MZ2, we obtained stable CTL clones that lyse specifically the autologous tumor cell line MZ2-MEL (Hérin et al. 1987). These CTL clones were used to select resistant MZ2-MEL antigen-loss variants, and this led to the conclusion that autologous CTL recognize several distinct antigens on the MZ2-MEL cells (Van den Eynde et al. 1989). One of these antigens was named MZ2-E.

Gene *MAGE-1*, which codes for antigen MZ2-E, was identified by a procedure based on gene transfection (van der Bruggen et al. 1991; Traversari et al. 1992b). It comprises three exons spread over 4.5 kilobases (kb) and shows no homology to any recorded gene. A nonapeptide encoded by *MAGE-1* combines with major histocompatibility molecule HLA-A1 to form antigen MZ2-E (Traversari et al. 1992a). Gene *MAGE-1* is expressed in tumors of various histological types, such as melanomas, lung, and breast carcinomas (van der Bruggen et al. 1991; Brasseur et al. 1992; Weynants et al. 1994). At first, no expression was found on a panel of healthy tissues (van der Bruggen et al. 1991), but more recently testis has been found to express *MAGE-1* (De Smet et al. 1994).

When a *MAGE-1* fragment was used as a probe on Southern blots prepared with human genomic DNA, it formed several hybridization bands of different intensities. This was the first indication that gene *MAGE-1* was a member of a gene family. The screening of a cDNA library

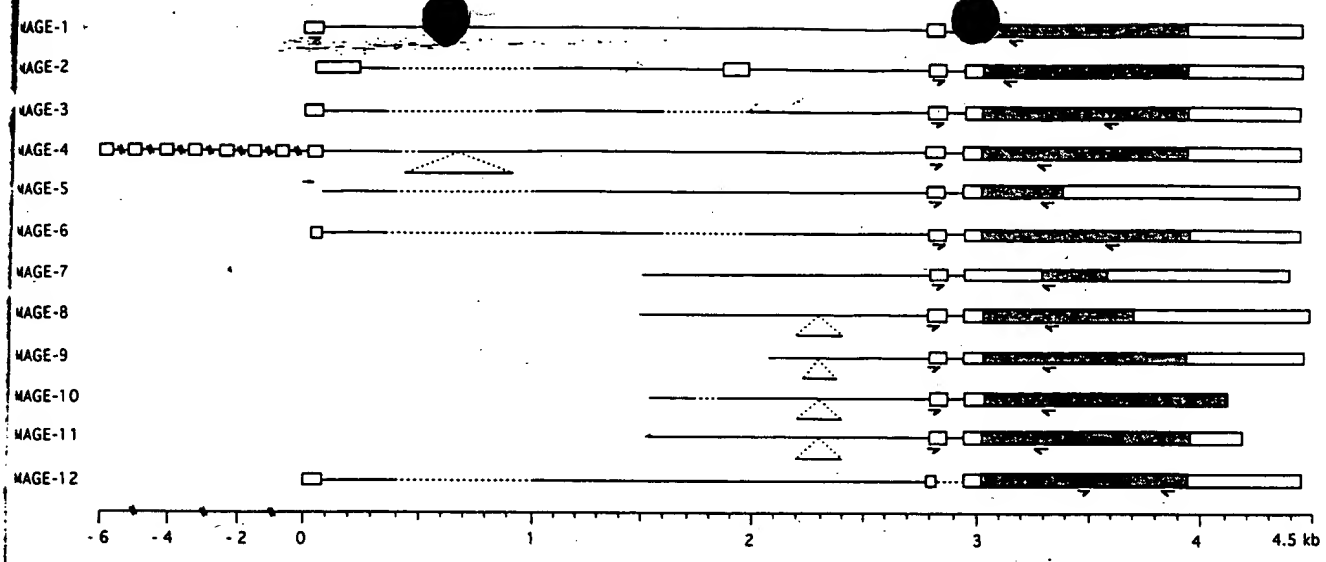


Fig. 1 Structure of 12 *MAGE* genes. The *lines* show the parts of the genes that have been sequenced. The sequence obtained for each gene was aligned to the sequence of *MAGE-1*. The 5′ end of the incomplete first introns corresponded to the end of the cosmid insert (*MAGE-9*) or to sequence that showed no homology with *MAGE-1* (*MAGE-7, 8, 10, 11*). Deletions are shown as *dashed lines*, insertions as lines *below* the main line. Exons are indicated as *open boxes*, with the large open reading frame as a *grey box*. In the last exon of *MAGE-1*, the region encoding the peptide of antigen MZ2-E is marked in *black*. *Arrows* represent the primers used to amplify specifically each *MAGE* cDNA

of MZ2-MEL confirmed this: in addition to the cDNA of *MAGE-1*, two closely related cDNAs were isolated. The corresponding genes were named *MAGE-2* and *MAGE-3* (van der Bruggen et al. 1991). By testing cosmid libraries with a *MAGE-1* probe, we identified nine additional genes belonging to the *MAGE* family. We describe here the structure, the chromosomal localization, and the pattern of expression of the 12 *MAGE* genes.

Materials and methods

Polymerase chain reaction (PCR) assays

RNA purification and cDNA synthesis were performed as described (Weynants et al. 1994). For amplification, 1/20 of the cDNA produced from 2 µg of total RNA was supplemented with 5 µl of PCR buffer (500 mM KCl, 100 mM Tris pH 8.3), 1 µl each of 10 mM dNTP, 25 pmoles of each primer, 3 µl of 25 mM $MgCl_2$, 1.25 units of *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, CT), and water to a final volume of 50 µl. Primers and PCR conditions to amplify *MAGE-1* and -2 are already described (Brasseur et al. 1992; De Smet et al. 1994).

The primers and lengths of PCR products for MAGE-3 to -12 were as follows: (sense, anti-sense)
MAGE-3: 5′-TGGAGGACCAGAGGCCCCC,
5′-GGACGATTATCAGGAGGCCTGC (725 bp)
MAGE-4: 5′-GAGCAGACAGGCCAACCG,
5′-AAGGACTCTGCGTCAGGC (446 bp)
MAGE-5: 5′-CTAGAGGAGCACCAAAGGAGAAG,
5′-TGCTCGGAACACAGACTCTGG (413 bp)
MAGE-6: 5′-TGGAGGACCAGAGGCCCCC,
5′-CAGGATGATTATCAGGAAGCCTGT (727 bp)
MAGE-7: 5′-CAGAGGAGCACCGAAGGAGAA,
5′-CAGGTGAGCGGGGTGTGTC (405 bp)
MAGE-8: 5′-CCCCAGAGAAGCACTGAAGAAG,
5′-GGTGAGCTGGGTCCGGG (399 bp)
MAGE-9: 5′-CCCCAGAGCAGCACTGACG,
5′-CAGCTGAGCTGGGTCGACC (391 bp)
MAGE-10: 5′-CACAGAGCAGCACTGAAGGAG,
5′-CTGGGTAAAGACTCACTGTCTGG (485 bp)
MAGE-11: 5′-GAGAACCCAGAGGATCACTGGA,
5′GGGAAAAGGACTCAGGGTCTATC (422 bp)
MAGE-12: 5′-GGTGGAAGTGGTCCGCATCG,
5′-GCCCTCCACTGATCTTTAGCAA (392 bp)

Amplification was performed in a TRIO-Thermoblock (Biometra, Göttingen, FRG) for 30 cycles (*MAGE-3, 4, 6, 12*) or 32 cycles (*MAGE-5, -7* to *-11*): 1 min at 94° C; 2 min at 65° C (*MAGE-5, -7* to *-12*), 68° C (*MAGE-4*) or 71° C (*MAGE-3* and *6*); 2 min (*MAGE-4*), or 3 min at 72° C (*MAGE-3, -5* to *-12*).

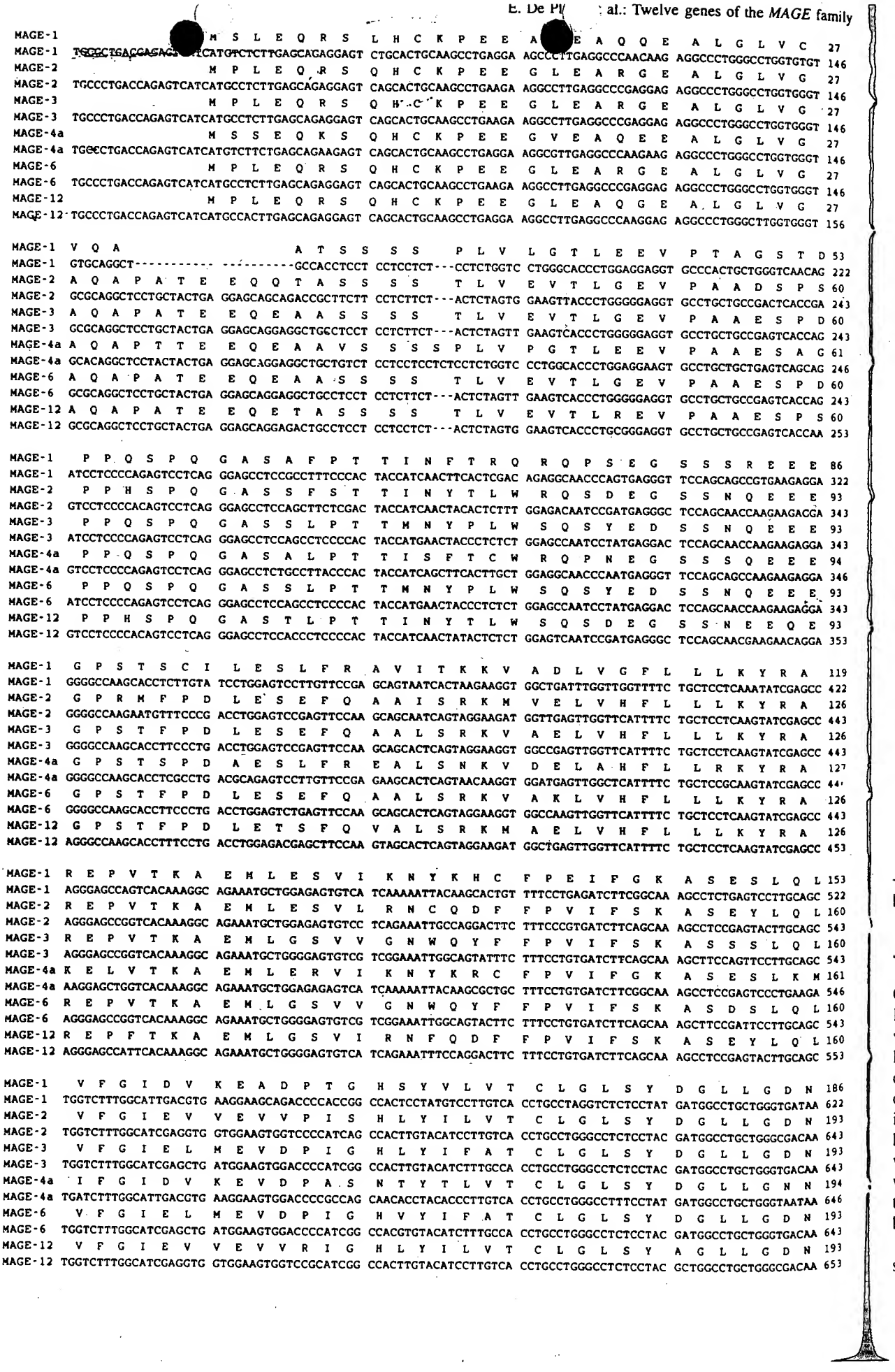
Analysis of somatic cell hybrids

The human-hamster hybrids designated with GM were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ), PgMe4 and those with A_3 were from P. Pearson (John Hopkins University). The human chromosome content of each somatic cell hybrid was established cytogenetically and by using mapped DNA probes at the time of DNA isolation.

Southern blots of genomic DNA were prepared by previously described methods (James et al. 1988). Hybridization with the labeled 2.4 kb *MAGE-1* genomic fragment was performed at 42° C for at least 16 h in 50% formamide, 5% dextran sulfate, 6× standard sodium citrate (SSC), 1% sodium dodecyl sulfate (SDS) and 0.1 mg/ml heterologous DNA. Filters were washed consecutively in 2× SSC, 0.1% SDS at room temperature for 15 minutes and twice in 0.1×SSC, 0.1% SDS at 67° C for 30 min each. Autoradiography was performed at −70° C for 7–10 days, using Fuji XR film.

Homology search

Sequence alignments of DNA or protein sequences with *MAGE-1* were performed with the GeneWorks computer program (Intelligenetics, Mountain View, CA). For DNA, we used parameters 30, 2, 10, and 4000 and for proteins, parameters 5, 25, 4, and 10. The computer search for sequence homology in Genbank was done with the Databank Search 1.1 and the BLAST programs, using the NCBI server.



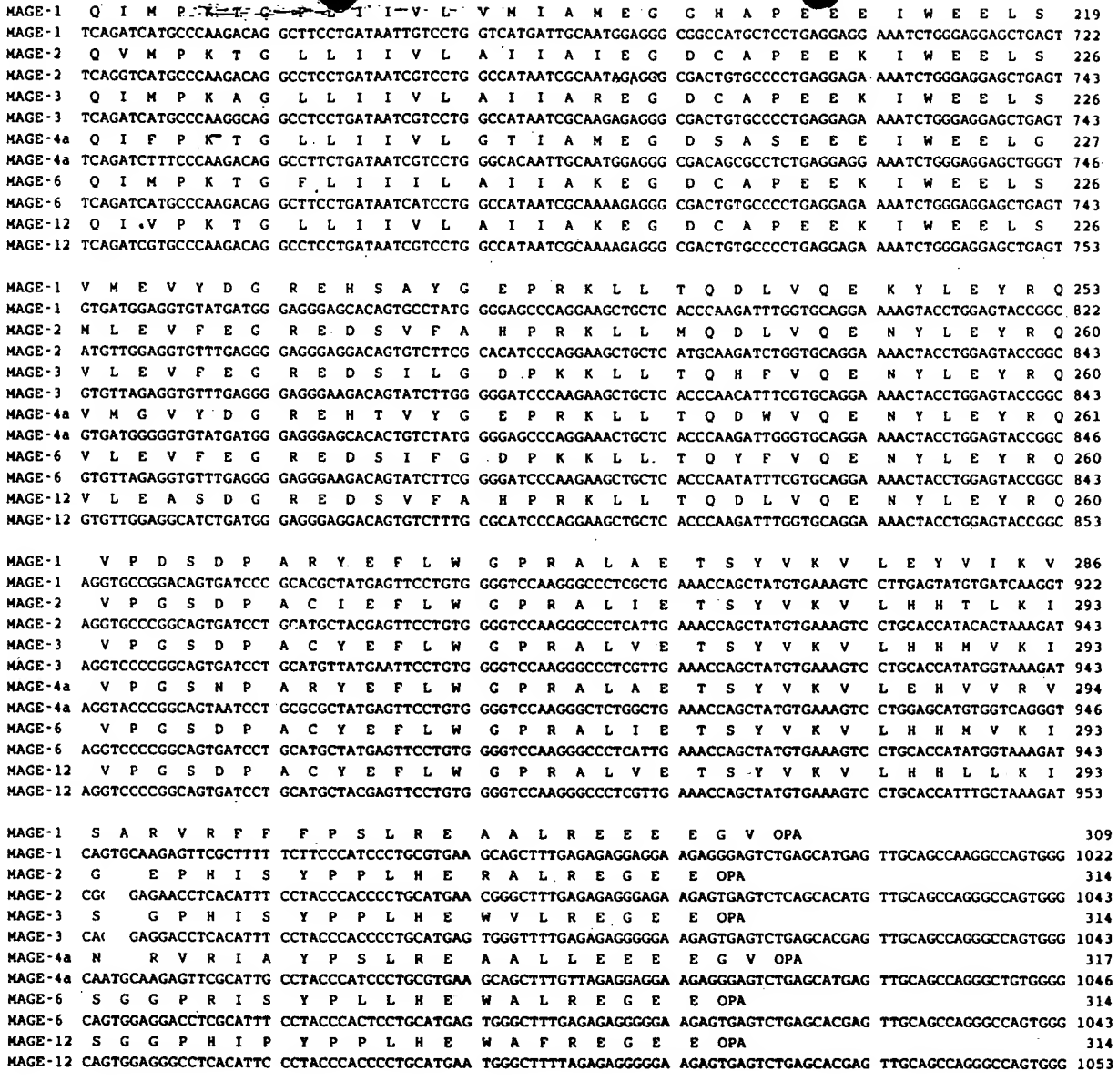


Fig. 2 Sequence alignment of the regions containing the largest open reading frame in the last exons of *MAGE 1, 2, 3, 4a, 6,* and *12*. Gaps introduced for optimal alignment are indicated by *dashes*. The numbering is relative to the first nucleotide of the last exon. The amino acid sequences of the putative proteins encoded by the six *MAGE* genes are represented

Results

Structure of the MAGE genes

Cosmid libraries were constructed in vector c2RB with DNA isolated from blood lymphocytes of patient MZ2 and with DNA of melanoma cell line MZ2-MEL. When these libraries were probed with a 2.4 kb *Bam* HI fragment that contained exons 2 and 3 of gene *MAGE-1*, 24 of 600000 cosmids were found to hybridize. In these cosmids, we identified *Bam* HI fragments of various lengths that hybridized with the *MAGE-1* probe. These fragments were subcloned and sequenced. Additional sequences were obtained by performing PCR sequencing directly on the cosmids. This led to the identification of 14 different homologous genes.

Gene *MAGE-1* was previously found to comprise two small exons followed by a large third exon containing the entire coding region (van der Bruggen et al. 1991). The exon-intron structure of genes *MAGE-2, 3, 4,* and *12* was established by aligning the genomic sequences with cDNA clones that showed complete identity with segments of these sequences. *MAGE-2* and *MAGE-3* cDNAs were obtained from melanoma subline MZ2-MEL 3.0 (van der Bruggen et al. 1991), *MAGE-12* cDNA from melanoma cell line, and LB373 and *MAGE-4* cDNAs were isolated from sarcoma cell line LB23, from placenta, and from testis. The two last exons of genes *MAGE-2, 3,* and *4* could be aligned completely with those of *MAGE-1* (Fig. 1). In *MAGE-12*, the sequence corresponding to the second

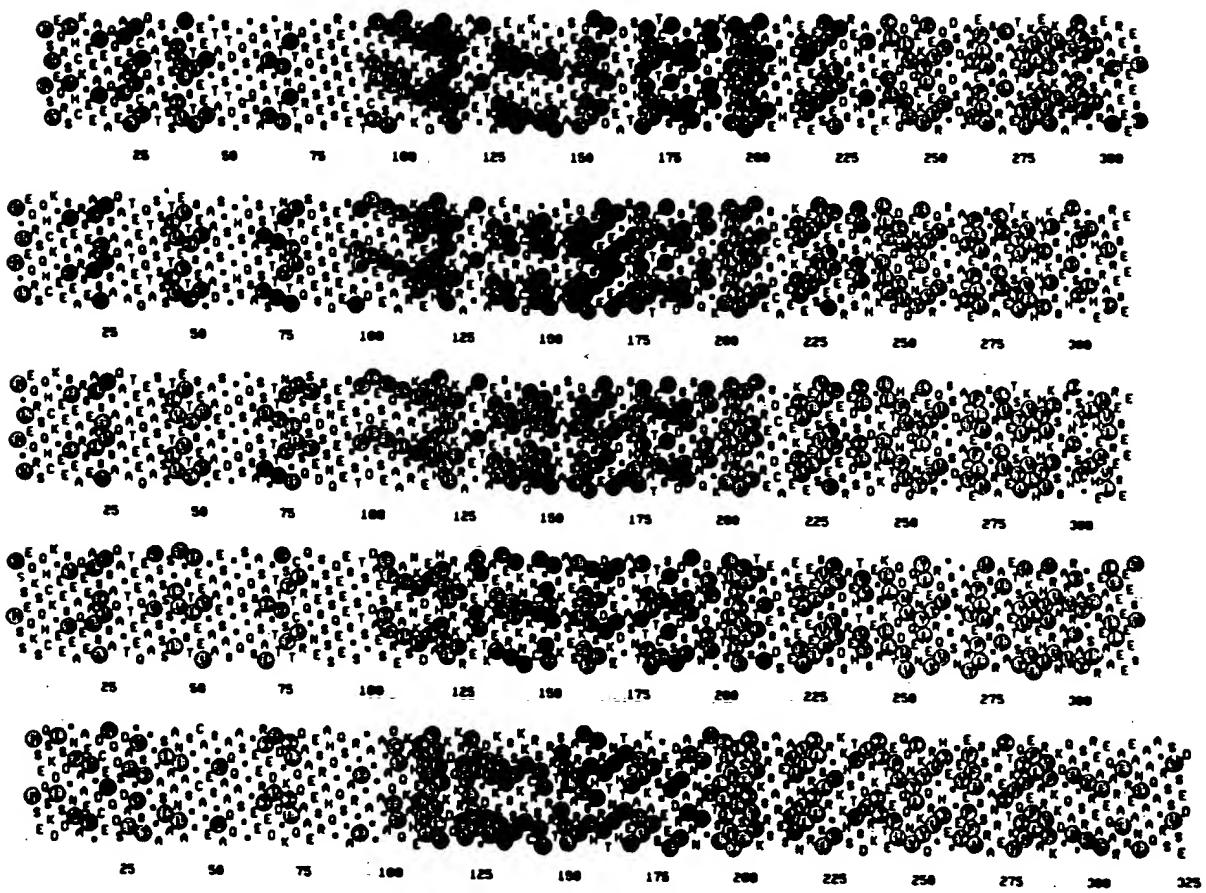


Fig. 3 α-helical patterns of hydrophobic cluster analysis (HCA) for *MAGE 1–4* and necdin amino acid sequences (Gaboriaud et al. 1987). The hydrophobic residues (VLIMYW) are presented as *dark circles*. The *arrow* shows the putative hydrophobic transmembrane domain conserved across the family

intron of *MAGE-1* is deleted so that a single exon corresponds to the two last exons of *MAGE-1*. The last exons of *MAGE-1, 2, 3, 4,* and *12* contain coding regions of nearly identical lengths (Figs. 1, 2). In the first intron, these genes display considerably more variation, even though homology remains unquestionable. The first introns of *MAGE-2, 3,* and *12* present large deletions relative to *MAGE-1*, whereas the first intron of *MAGE-4* contains a large unrelated insert (Fig. 1). Remarkably, *MAGE-2* has an additional exon homologous to a sequence located in the first intron of *MAGE-1*. This additional exon is not conserved in *MAGE-12*, which is 90% identical to MAGE-2. The disposition of the initial exons of the five *MAGE* genes also shows many differences (Fig. 1). The first exon of *MAGE-2* extends 175 bases beyond that of *MAGE-1, 3,* and *12* and this extension is homologous to the initial intron region of *MAGE-1, 3,* and *12*. In *MAGE-4*, we identified eight sequences dispersed over 6 kb and serving as alternative first exons. *MAGE-4* cDNAs isolated from sarcoma LB23, placenta, and testis had different 5′ ends corresponding to each of these sequences, all spliced to the same second exon. Only for their last 18 nucleotides are these alternative first exons homologous to the first exon of *MAGE-1*.

The sequence of gene *MAGE-6* was found to be 99% identical to that of *MAGE-3*. And the few differences between the two genes were all located in the last exon of *MAGE-3*. To establish the exon-intron structure of *MAGE-6*, we carried out reverse transcription and PCR amplification on RNA of melanoma cell line LE MD3A which expresses *MAGE-6* and not *MAGE-3*. Primers were chosen in the sequence of *MAGE-6* that corresponded to exons 1 and 3 of *MAGE-3*. The sequence of the cDNA fragment delineated the structure shown in Figure 1.

The structure of genes *MAGE-5* and *7–11* has not yet been completely defined because no cDNA clones have been obtained up to now. The two last exons of these genes were tentatively delineated on the basis of sequence similarity with exons 2 and 3 of *MAGE-1*. For genes *5* and *8–11*, the boundaries of these exons were confirmed by sequencing PCR products obtained from cDNA with primers located in each exon.

The percentage of identity of the last exon of *MAGE-1* with that of the other *MAGE* genes varies between 64% and 85%. Some genes are very closely related. For pairs of

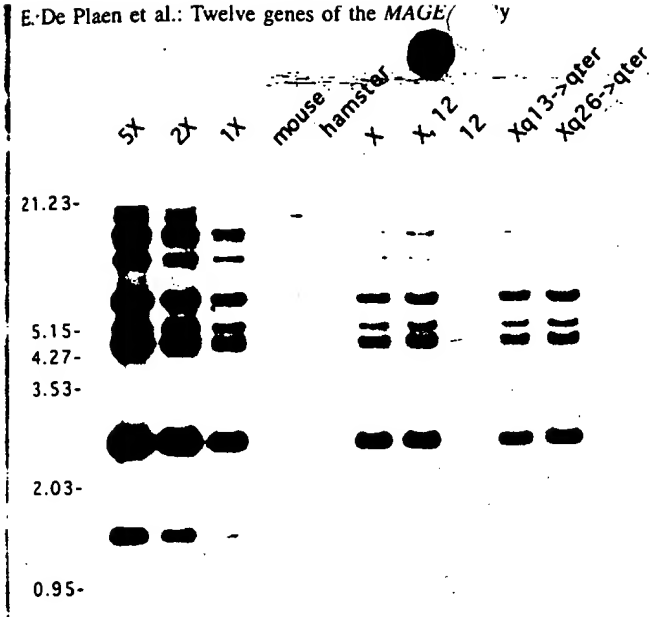


Fig. 4 Southern blot of *Bam* HI digests of genomic DNA from hamster, mouse, human cell lines and hamster-human somatic hybrid cell lines. Lane 1: somatic hybrid line GM06061B (5 human chromosomes X); lane 2: normal human female (2 chromosome X); lane 3: normal human male (1 chromosome X); lane 4: mouse DNA; lane 5: hamster DNA; lane 6: hybrid cell line GM06318B containing human chromosome X; lane 7: hybrid cell line GM07301 containing human chromosome 12 and X; lane 8: hybrid cell line GM10868 containing human chromosome 12; lane 9: hybrid cell line GM10095 containing only the Xq13-qter portion of chromosome X; lane 10: hybrid cell line GM10482 containing only the Xq26-qter region of chromosome X. The 2.4 kb genomic fragment containing exons 2 and 3 of *MAGE-1* was used as probe

genes *4a/4b* and *5a/5b*, the identity exceeds 99%. As shown below, these pairs are alleles. We compared the sequences of all the *MAGE* genes with known sequences in databases and found no significant homology.

MAGE proteins

Most of the putative MAGE proteins are 309–319 amino-acids long. MAGE proteins 2–6 and 8–12 have 57%–77% identity with *MAGE-1*. *MAGE-7* was not included in this comparison, since this gene was not found to be transcribed (see expression of *MAGE* genes) and since the largest open reading frame in this gene is not in phase with those of the other *MAGE* genes (Fig. 2). The MAGE proteins are devoid of signal sequences and contain a potential transmembrane domain (Fig. 3) that, because of its small size, may function only in association with the transmembrane domain of another protein. A computer search of the Protein Sequence Database revealed a moderate homology (31% identity) of MAGE-10 protein with mouse protein necdin. Necdin has been reported to be a nuclear protein, expressed in neurally differentiated embryonal carcinoma cells and in the brain of adult mice (Maruyama et al. 1991). We used the hydrophobic cluster analysis [(HCA) (Gaboriaud et al. 1987)] to compare the amino acid sequences of *MAGE 1–4, 6, 8–12*, and nec[illegible] (Fig. 3). This analysis demonstrated a remarkable conservation of the main hydrophobic regions, suggesting conservation of function.

Low polymorphism of MAGE genes

It was important to examine the degree of polymorphism of *MAGE* genes, since different *MAGE* alleles may produce different antigenic peptides. Several lines of evidence suggest that *MAGE* genes show little variation from one individual to another. A Southern blot of genomic DNA, which was obtained from blood lymphocytes of ten individuals, was probed with a labeled *MAGE-1* genomic fragment. We observed that the pattern of 13 hybridizing fragments was conserved, except for the presence of an additional band for one individual (De Smet et al. 1994; data not shown). For genes *MAGE-4a, 4b, 3, 5a, 5b*, and *6*, the sequences of PCR products derived from lymphocytes of patient MZ2 were completely identical to the sequences of products derived from somatic cell hybrid GM07301, whose human chromosome X comes from another individual. From genomic DNA prepared from blood lymphocytes of 15 individuals, we also amplified a fragment of gene *MAGE-3* that contains a sequence encoding an antigenic peptide presented by HLA-A1 (Gaugler et al. 1994). A sequence of 400 bases surrounding the sequence of the antigenic peptide was obtained for each of the PCR products. The 15 sequences were completely identical.

Chromosomal localization

The chromosome bearing the *MAGE* genes was identified by Southern blot analysis of the DNA of a panel of hamster-human somatic cell hybrids. The 2.4 kb genomic segment containing the two last exons of *MAGE-1* was used as a probe under conditions of stringency such that no cross-hybridization was observed with hamster DNA. The presence of the *MAGE* sequences in the hybrid cell lines was concordant only with the presence of human chromosome X (Table 1; Fig. 4). Cell line GM06061B contains five X chromosomes; the analysis of its DNA showed the expected dosage effect (Fig. 4).

We then examined whether all 12 *MAGE* genes are located on this chromosome. To this end, we identified sets of PCR primers that provided strict specificity for each of *MAGE 1–12*, as verified by the sequencing of the PCR products. PCR assays specific for *MAGE-1–12* were performed on DNA of hybrid cell line GM10868 containing human chromosome 12 and of line GM07301 containing human chromosomes 12 and X. All were negative with the former and positive with the latter, indicating that all 12 *MAGE* genes are located on chromosome X.

Hybrid cell line GM10095 was derived from a human cell line having a translocation t [(X;9) (q13;q34)] between chromosome X and chromosome 9. The DNA of this hybrid, which contains only the X/9 translocation chromosome and therefore only the q13-qter region of the

Table 1 Segregation of *MAGE-1* with human chromosomes in human – hamster hybrid cell DNA. + = chromosome present; – = chromosome absent; ± = very faint bands, indicating that only a small percentage of the cells contained the chromosome (not included in calculation of percent discordancy); 1,2 – GM09142 contains only part of chromosomes X and 21, der t(X;21)(p21;p12); 3,4 – GM10095 contains only part of chromosomes X and 9, der 9 t(X;9)(q13;q34); 5 – PgMe4 contains a deleted chromosome 2 and is missing 2p23–24; 6 – A_3G1 contains only the q arm of chromosome 6

Hybrid	*MAGE-1*	Human chromosome																							
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y
GM06317	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+
GM06318B	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
GM07300	+	–	–	–	–	–	+	–	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	+	–
GM07301	+	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	+	–
GM08854	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–
GM09142	–	–	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–	2	–
GM10095	+	–	–	–	–	–	–	–	–	3	–	–	–	–	–	–	–	–	–	–	–	–	–	4	–
GM10115	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
GM10156B	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
GM10253	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
GM10322	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–	–
GM10478	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–
GM10479	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–
GM10498	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–
GM10567	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–
GM10611	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
GM10612	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–
GM10629	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
GM10791	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
GM10880	–	+	–	–	–	–	–	–	–	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–
GM10888	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–
A_3ADA ID12	–	+	–	+	+	+	–	–	+	–	–	–	+	+	+	–	+	–	–	–	+	+	–	–	+
A_3ADA6F5	+	+	+	–	–	+	+	+	+	–	–	–	+	+	+	+	–	+	+	+	–	+	+	+	+
A_3ADA13	+	+	–	+	+	–	–	+	+	–	–	+	+	+	+	+	±	–	–	+	–	+	–	+	+
A_3ADA14	–	–	–	+	+	+	–	–	+	–	+	+	+	+	+	±	–	–	–	–	+	+	+	–	+
A_3G1	+	–	–	+	+	–	6	–	±	+	+	–	–	–	–	±	–	+	–	±	+	–	±	+	+
A_3HR20	–	–	–	+	+	+	+	–	–	+	–	+	–	–	+	–	–	+	–	–	–	–	±	–	–
P_gMe4	+	–	5	+	–	–	+	+	+	–	–	+	+	+	+	+	–	+	–	–	±	+	–	±	+
Number of concordant hybrids	(+/+)	2	1	3	2	1	3	3	4	2	1	3	4	3	3	3	0	3	1	2	1	3	1	8	4
	(–/–)	17	19	14	14	15	18	18	16	17	18	17	17	16	14	18	17	17	19	18	16	14	15	18	17
Number of discordant hybrids	(+/–)	6	6	5	6	7	3	5	3	6	7	5	4	5	5	4	7	5	7	5	6	5	6	0	4
	(–/+)	2	0	5	5	4	2	1	3	2	1	2	2	3	5	0	2	2	0	1	3	5	3	0	2
Percent discordancy		22	23	38	37	41	24	22	23	30	30	26	22	30	37	16	33	26	27	23	35	37	36	0	26

X chromosome, hybridized to the *MAGE-1* genomic probe. Hybrid GM10482, which is derived from a t [(X;11)(q26;q23)] cell line carries only the q26-qter region of the X chromosome. Its DNA also hybridized to the *MAGE-1* probe and the pattern of hybridizing fragments was the same as that observed with the entire X chromosome (Fig. 4). We conclude that most if not all *MAGE* genes are located in the terminal region of the long arm of chromosome X.

Additional evidence suggested that most *MAGE* genes are closely linked. Several cosmids were found that carried two *MAGE* genes. This was observed for *MAGE-3* and *MAGE-2*, *MAGE-6* and *MAGE-2*, *MAGE-5a/b* and *MAGE-10*, *MAGE-7* and *MAGE-9*.

Because some pairs of *MAGE* genes were found to be almost identical, there was a possibility that these pairs represented alleles of one *MAGE* locus. This appears to apply to pairs of genes *MAGE-4a/4b* and genes *MAGE-5a/5b*, because the sequencing of PCR products revealed the presence of only *4b* and *5b* in cell hybrid GM07301. On the contrary, *MAGE-3* and *MAGE-6* sequences, which are also 99% identical, were both found in hybrid GM07301 which contains a single X chromosome. They belong therefore to different loci. This was confirmed by PCR assays on genomic DNA of EBV-transformed B cells of five male individuals: both *MAGE-3* and *MAGE-6* were found in the genome of these cells.

Expression of MAGE genes

Because the sizes of the various *MAGE* mRNAs are similar, one cannot evaluate on a northern blot the expression of individual *MAGE* genes in various normal and tumoral tissues (van der Bruggen et al. 1991). To measure the expression of these genes, we therefore resorted to oligodT primed reverse transcription of total RNA followed by PCR amplification with primers specific for each *MAGE* gene. For *MAGE-1–11*, effective PCR primers could be identified in different exons, so that the PCR products derived from RNA can be distinguished by size from those that might be produced by contaminating DNA.

Table 2 Expression of *MAGE-1, 2, 3, 4, 6,* and *-12* by tumors and normal tissues. RNA from tumor cell lines (¶), tumor samples (**) and normal tissues were tested by RT-PCR for the expression of *MAGE* genes. PCR primers were chosen as indicated in methods. For *MAGE-12*, PCR amplification of RNA in the absence of reverse transcription indicated that in our conditions the contamination by genomic DNA was negligible. The level of expression evaluated by band intensity of PCR products fractionated in agarose gels is represented by +++, ++, +. Absence of product is indicated by –

	MAGE 1	*MAGE 2*	*MAGE 3*	*MAGE 4*	*MAGE 6*	*MAGE 12*
COLON CARCINOMAS						
MZ-CO-2 ¶	++	++	–	–	–	+
SK-CO-11 ¶	–	++	+++	–	+	++
LB150**	–	–	–	+	–	–
HSR 320 ¶	–	+++	+++	+	++	+++
LEUKEMIAS						
K562 ¶	–	++	+++	–	++	+++
MELANOMAS						
MI10221 ¶	+	+++	+++	+++	+++	+++
MZ2-MEL 3.0 ¶	+++	+++	+++	–	+++	+
LB265**	–	++	–	–	–	+
LG7**	–	++	–	–	–	–
LG11**	++	++	++	–	–	+++
LB271**	–	++	+++	–	++	+++
LUNG CANCERS						
LB178 (NSCLC)**	++	–	–	+++	–	–
LB175 (NSCLC)**	++	++	+++	+++	++	+++
LB11 (SCLC) ¶	++	+++	+++	–	–	+++
LB12 (SCLC) ¶	–	+++	+++	–	–	+++
SARCOMAS						
LB23 ¶	–	–	–	++	–	–
LB408**	–	–	–	++	–	–
LB258**	+	++	+	–	–	++
BREAST CARCINOMAS						
LB280**	++	–	++	–	–	+
LB284**	++	++	++	+	–	++
Stomach	–	–	–	–	–	–
Lung	–	–	–	–	–	–
Breast	–	–	–	–	–	–
Colon	–	–	–	–	–	–
Skin	–	–	–	–	–	–
Uterus	–	–	–	–	–	–
Testis	++	++	++	++	++	++
Thymocytes	–	–	–	–	–	–
EBV-lymphocytes	–	–	–	–	–	–
Foetal liver	–	–	–	–	–	–
Foetal brain	–	–	–	–	–	–
Placenta LB694	–	–	+	+++	–	–

MAGE-1, 2, 3, 4, 6, and *12* showed significant expression in a number of tumors of various histological types. Table 2 presents a representative sample of positive tumor cells. The level of expression of these *MAGE* genes was similar to that observed with *MAGE-1* in the MZ2-MEL cell line, which expresses a *MAGE-1*-encoded antigen. In a MZ2-MEL cDNA library of 100000 clones, we found *MAGE-1* clones at a frequency of 1/4000, corresponding to approximately 50 copies/cell.

MAGE-5, 8, 9, 10, 11 were very weakly expressed in all the samples that we examined; we estimated that the amount of RNA of these genes represented less than 1% of that of the highly expressed genes. We were unable to retrieve in a MZ2-MEL library a single cDNA corresponding to *MAGE-5*, suggesting the presence of <2 copies/cell. *MAGE-7* was not transcribed at all in the 95 tumor samples tested.

A panel of normal adult tissues and some tissues from >20-week-old fetuses were also tested for the expression of *MAGE* genes (Table 2). All were negative, with the exception of testis and of placenta. Testis expresses all *MAGE* genes, except *MAGE-7*. Placenta expresses *MAGE-3, MAGE-4,* and *MAGE-8–11*.

Discussion

The *MAGE* gene family comprises at least 12 closely related genes, which are located on the long arm of chromosome X. Because we have analyzed only 24 cosmids carrying *MAGE*-related sequences, it is possible that a few additional *MAGE* genes remain to be found. Several of the MAGE genes are very closely linked and the whole *MAGE* family may be located in a single complex. By analyzing YAC clones, it should be possible to precisely determine the relative position of each *MAGE* gene.

The lengths and sequences of the two last exons and of the last intron of the *MAGE* genes are conserved, as might be expected for regions located close to the coding part of the gene. Throughout the *MAGE* family and necdin, there is considerable conservation of hydrophilic and hydrophobic regions, suggesting that the proteins produced by all these genes may exert very similar functions. At the present time, however, there is no indication regarding this function.

Much more variation is observed in the initial part of the genes, with large deletions, insertions, and even an additional exon occurring in the first intron of *MAGE-2*. The 5′ end of gene *MAGE-4* is remarkable, since it contains

several alternative first exons. The promoter regions of the various *MAGE* genes are very different (De Smet and co-workers, in preparation). We suggest therefore that successive duplications of a *MAGE* gene into a large gene family has enabled the same function to come under different transcriptional controls resulting in very specific regional and temporal expressions.

MAGE genes *1, 2, 3, 4, 6,* and *12* have been found to be significantly expressed in a number of tumors of different histological types. No expression of these genes was found in a panel of normal tissues, with the exception of testis and placenta. It should be possible to identify the positive testis cells with antibodies directed against the MAGE proteins.

Because the *MAGE* genes do not seem to be expressed in normal tissues except testis and placenta, they all have the potential to code for antigens that could be targets for specific anti-tumor T-lymphocyte responses. For viral proteins such as the influenza nucleoprotein, several different peptides corresponding to various sites of the protein have been found to combine with various class I molecules to form antigens recognized by different CTL (McMichael et al. 1986; Taylor et al. 1987). It is therefore likely that various regions of the different MAGE proteins can contribute peptides combining with various HLA class I molecules. Evidence supporting this has been obtained recently. A MAGE-1 peptide that is different from the peptide that forms antigen MZ2-E by combining with HLA-A1, was found to bind to a HLA-C molecule to form an antigen recognized by an autologous CTL (van der Bruggen et al. 1994). MAGE-3 codes for an antigenic peptide that binds to HLA-A1 to form an antigen that is distinct from the MZ2-E antigen (Gaugler et al. 1994). It therefore appears likely that several other MAGE antigens presented by various HLA molecules will be identified. One approach to identify them involves the use of existing anti-tumor CTL whose HLA restricting element is known. They can then be tested for stimulation by COS cells cotransfected with *MAGE* cDNAs and with the relevant *HLA* gene. Another approach is based on the observation that peptides that bind to certain HLA have "consensus" residues in some positions (Falk et al. 1991; Jardetzky et al. 1991). Consensus peptides coded by the sequences of the various *MAGE* genes could be identified, synthesized, and tested for their ability to bind to the relevant *HLA* (Hill et al. 1992; Houbiers et al. 1993). Using efficient antigen-presenting cells, it may be possible to obtain CTL directed against some of these peptides.

Attempts will be made to immunize cancer patients against MAGE-encoded antigens. For this, it will be necessary to identify the patients whose tumor expresses a known antigen. This can be done by HLA typing and by reverse transcription and PCR amplification of the RNA of a small tumor sample, to identify the *MAGE* genes expressed in the tumor. This approach will probably be reliable because of the very low level of polymorphism of *MAGE* genes. Considering the diversity of the various *MAGE* genes, it will be essential to use PCR primers that properly distinguish the various *MAGE* genes. We believe that this can be achieved with the primers described here.

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